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Department of Anatomy and Department of Pathology, University of Washington

STUDIES ON THE NATURE OF PLANARIAN CONNECTIVE TISSUE*

By

KNUD JØRGEN PEDERSEN**

With 18 Figures in the Text

(Received November 3, 1960)

The last decade or two has witnessed an extensive revival of the interest in connective tissue. The significance of this tissue for normal and pathological physiological processes in the organisms has gained appreciation. Most studies so far have been centered about mammalian connective tissue. Very little work has been conducted on the connective tissue in lower vertebrates and in invertebrates. This is regrettable because a comparative approach to cytological and histological problems is generally of great value. Many general problems are often more easily attacked and comprehended from a broad background of comparative facts. Moreover, the variety of the connective tissue in the animal kingdom presents an intriguing challenge to the histologist.

Besides the outstanding function of connective tissue in support at the macro- and microscopical level of organization, it is also of great importance as a depot for metabolites, for exchange of materials between cells and in inflammatory and regenerative processes.

The present paper deals with some observations on the connective tissue of planarians [Phylum: Platyhelminthes, class: Turbellaria, order: Tricladida (HYMAN 1951)]. The remarkable regenerative capacity of these animals is well known and is closely related to properties of their connective tissue. Moreover, the systematic position of turbellarians in the animal kingdom and their significance for phylogenetic problems (REISINGER 1959) makes a study of their connective tissue desirable. The phylum to which they belong is generally considered to be the lowest one where an extensive development of connective tissue has taken place due to the formation of a "true" mesoderm, an entomesoderm (HYMAN 1951).

The subject of the planarian connective tissue or parenchyma (see a following section on nomenclature) is one of notorious confusion. An historical evaluation

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is accordingly difficult. PRENANT (1922), in his important monograph on the parenchyma of *Platyhelminthes*, states this in the following way: »Tous les auteurs n'emploient pas les mêmes termes; il est rare qu'ils se rallient purement et simplement á une interprétation déjá exprimée; certaines descriptions sont, de plus, confusément enoncées.« Little histological work on the planarian parenchyma has been published since PRENANT's excellent study. The reader is referred to this for a brief review of the historical aspects.

Some of the important classical contributions to the understanding of the planarian parenchyma will be evaluated later on in relation to the findings in the present paper.

Material and methods

Two species were employed. That used most extensively was the unpigmented *Planaria vitta*, which has been the subject of two preceding papers on planarian cytology (PEDERSEN 1959a, 1959b). Cloned individuals were used. The second species was *Dugesia tigrina (Planaria maculata)*. The combined use of these two species was found to be a fortunate coincidence, as the information obtained on the parenchyma of one supplemented that from the other. *Planaria vitta* was most useful in the light microscopical study of the parenchyma, but was unsuitable for certain electron microscopical techniques, whereas *Dugesia tigrina* was convenient for electron microscopy and enzymatic histochemical studies.

Histological and histochemical methods. Most of the methods employed have previously been described in detail (PEDERSEN 1959a, 1959b). However, some additional methods were used in the present study. The enzymatic histochemical studies were much facilitated by the application of cryostat method (the Pearse-type cryostat was used). Some animals were quenched in iso-pentane cooled in liquid nitrogen. From these fresh-frozen sections were prepared in the cryostat. Other animals were fixed in formalin-calcium, embedded in gum-acacia-sucrose as previously described and cut in the cryostat.

For demonstration of alkaline phosphatase, BURSTONE's method was employed (BURSTONE 1958a). For acid phosphatase both GOMORI's (1952) and BURSTONE's (1958b) methods were used. Succinic dehydrogenase was demonstrated according to NACHLAS et. al (1957). Cytochrome oxidase was demonstrated by BURSTONE's method (1959).

Methods for electron microscopy. In preliminary studies the animals were fixed in 1% osmium tetroxide after PALADE and embedded in methacrylate. However, a striking improvement in the preservation of the tissue was obtained by turning to fixation in osmium tetroxide buffered by s-collidine (BENNETT and LUFT, 1959) followed by embedding in the epoxy resin "Epon" (LUFT in press). The planarians were fixed in 2—3% osmium tetroxide for 2—4 hours and cut into smaller pieces during the dehydration, which was kept short (1 hour or less). The sections were cut on a PORTER-BLUM microtome using glass knives. Sections displaying light yellow to gray interference colors were selected and picked up on copper grids (200 mesh) covered by a thin carbon film. The grids were first examined in a phase contrast microscope with the high power dry lens. This allowed a satisfactory judgment of the quality of the sections. Most sections were stained in 2% uranyl acetate for 2—4 hours (WATSON 1958). The grids were examined with a RCA 2 A or 2 C electron microscope provided with special external lens power supplies.

Terminology

The terminology of the connective tissue in planarians is somewhat variable and often confusing. According to CHICHKOFF (1892) the first term employed to planarian connective tissue was "parenchyma". The term "reticulum conjonctif" was used by HALLEZ (1879) (v. GRAFF 1912—1917). "Mesenchyme" was used by IIJIMA (1884) and UDE (1908) and is also the one employed in the most recent extensive monograph on planarian morphology (HYMAN 1951).

In the present paper the term "parenchyma" will be used, partly because of its priority and partly because of its lack of embryological implications (see BRESSLAU 1928—1933).

In this section an attempt will be made to define the components of planarian connective tissue. The term "parenchyma", as applied in planarian histology, is defined as the tissue penetrating and binding together all structures between the epidermis and gastrodermis. At the light microscope level, using classical histological methods, two components can be resolved in the parenchyma. First are the conspicuous free cells, the *neoblasts*, which are well demarcated, rather small and exhibit a strongly basophilic cytoplasm due to a high content of ribonucleic acid (Pedersen 1959b). These cells play a decisive role in regeneration processes and are responsible for the high regenerative capacity of planarians. Second, the parenchyma contains an acidophilic mass with scattered nuclei, not exhibiting any distinct cell limits. This mass, which has been the subject of much controversy and misunderstanding because of lack of suitable methods for its study, constitutes, according to the interpretation given in the present paper, the cytoplasm of the "*fixed*" *parenchyma cells*.

The term "connective tissue" as applied to planarian histology will comprise the parenchyma proper (neoblasts plus "fixed" parenchyma cells) together with their possible derivatives, such as the subepidermal basement membrane.

For convenience the rather vague term "parenchyma" will be used in the following description of the light microscopical findings as denoting the acidophilic mass, the nature of which is the main theme of the present paper.

It should be noted that the term "parenchyma" applied to planarians and to several other invertebrates is at variance with the meaning of "parenchyma" as employed in vertebrate histology where it is used in the sense of "the essential specialized substance of an organ as distinguished from the stroma" (Montagna 1959).

In general histology the word "parenchyma" is used to indicate a mesenchyme which is completely cellular and does not contain any ground substance (Prenant, Bouin and Maillard 1904). These authors mention the parenchyma of flatworms in some detail and stress the potentialities of this tissue.

Light microscopical observations

All students of planarian histology have found it very hard to give a sound interpretation of the parenchyma based on the observation with the light microscope. As phrased by Hyman (1951): "The histological construction of the mesenchyme is exceedingly difficult to ascertain and it is not yet thoroughly understood." All studies of the histology of the parenchyma contain comments betraying difficulties involved. It is symptomatic that the very experienced and skillfull morphologists of the last century and of the beginning of the present century came to diametrically opposed conclusions as to the nature of the parenchyma.

As conventional histological technique has not progressed essentially since that time, though theoretical understanding of the procedures involved has advanced, it seems as if the classical histological approaches to this difficult problem have severe shortcomings. This was already envisaged by Böhmig (1886) who, in his study of the parenchyma in rhabdocoel turbellarians, used isolation procedures, and by Prenant (1922), who employed nearly all histotechnical procedures known at that time, including enzymatic digestion, and stressed the significance of such a multiple approach to the problem.

There are several reasons for these failures of the classical approaches. First, the parenchyma is difficult to fix properly and the artifacts produced are further enhanced by paraffin embedding. Second, in at least some planarian species (for example, *Planaria vitta*), the parenchyma contains numerous lipid droplets which are often large. Unless special precautions are taken, these will be dissolved and give rise to many irregular spaces which may be mistaken for evidence of

intercellular spaces. Their thin collapsed walls may be mistaken for fibrils. Third, it is important to use thin sections. This precludes the use of celloidin embedding and frozen sections. Fourth, the serious shrinkage involved in paraffin embedding gives rise to artifacts. Fifth, none of the classical staining methods give reproducible and meaningful histological pictures. Even at best, they usually allow only ambiguous interpretations. Sixth, in the words of GELEI (1912), considered to be one of the keenest observers of planarian histology [cited after

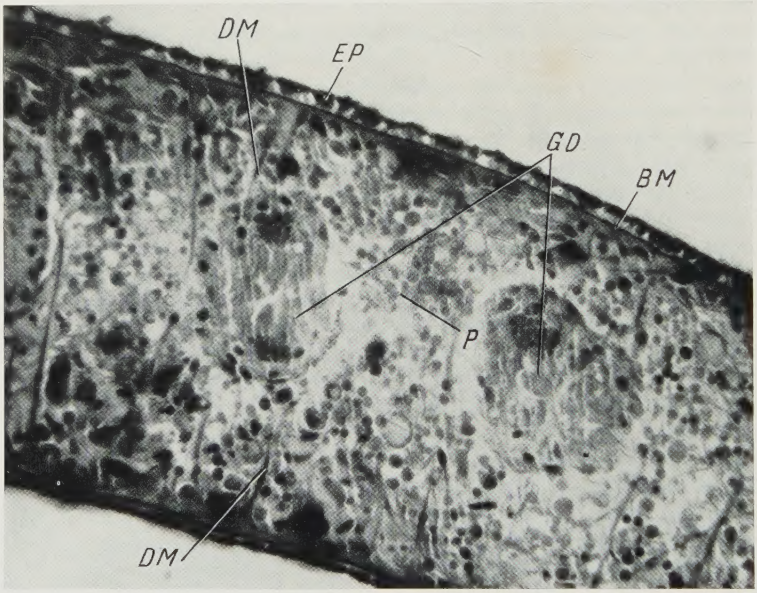

Fig. 1. Sagittal section of *Planaria vitta*. The general organization of the animal is seen. The epidermis (*EP*), basement membrane (*BM*), subepidermal muscle, dorso-ventral muscle (*DM*) and gastrodermis (*GD*) is seen. The tissue between these structures is mainly the parenchyma (*P*). Azan. Helly, 280 ×

TÖRÖK and RÖHLICH (1959)]: "...here ... the various sorts of tissues ... penetrate each other in all directions and it is very difficult, many times, to distinguish the cell types too."

In view of these difficulties, only one object will be pursued in the following presentation of light microscopical observations: this is merely to provide a generalized description of the parenchyma without attempts at interpretation. It is realized that the application of modern cytological technique for light microscopy aids considerably in gaining an understanding of the nature of the parenchyma, but that only an integration of these observations with those based on the electron microscope permits a really valid interpretation.

Of the two species investigated with the light microscope, *Planaria vitta* has received the most attention. *Dugesia tigrina* has been the object of such studies to only a minor degree. Unless otherwise specified, statements given will be based on observations on *Planaria vitta*. However, the basic features of the parenchyma are very similar in both species. At this point it seems pertinent to mention the main difference between the parenchyma in *Planaria vitta* and *Dugesia tigrina*. While the parenchyma in *Planaria vitta* is loaded with lipid droplets of varying

size, this is not the case with *Dugesia tigrina* in similar nutritional states. Here fat droplets are only rarely seen.

For general histological studies, fixation for a short time in HELLY'S or ZENKER'S fluid was valuable. Fixation in BOUIN'S or CARNOY'S fixatives or in buffered formalin did not give good preservation of the morphology. The best fixation was obtained by treatment with buffered osmium tetroxide. Freeze-drying came next to this in excellency of preservation.

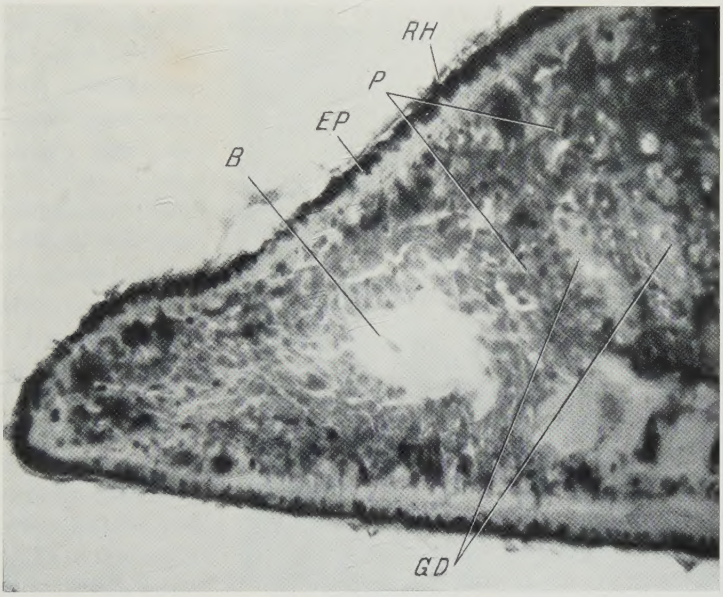

Fig. 2. Sagittal section through the head *(Planaria vitta)*. The strongly PAS-positive parenchyma (*P*) is seen between the virtually negative brain (*B*) and digestive tract (*GD*). Frozen-dried. Allochrome. 280 ×. (*RH* rhabdite, *EP* epidermis)

After HELLY and ZENKER fixation, the most generally useful stains were HEIDENHAIN'S azan method (Fig. 1), LILLIE'S allochrome method (see below) or LILLIE'S azur A- eosin B method at certain p_H-values (PEDERSEN 1959b).

The parenchyma is most complex in the subepidermal and in the head region and least in the more central regions (for example, between the diverticulae of the digestive tract). In the subepidermal region the picture is confusing because of the presence of many unicellular gland cells and the strongly developed subepidermal musculature. *Planaria vitta* is not pigmented, while the picture is further complicated in *Dugesia tigrina* by the presence of pigment cells, especially occurring in the dorsal subepidermal region. The free parenchyma cells, the neoblasts, which are easily visualized, have previously been studied (PEDERSEN 1959b). They are conspicuous because of their strongly basophilic cytoplasm, relatively large nuclei and prominent nucleoli. The appearance of the tissue left over after the organ systems, the neoblasts and the gland cells have been accounted for is a moderately acidophilic mass, often of a fibrillar and coarse to fine vacuolar appearance. It is most easily visualized in sagittal sections between the diverticulae of the gastrodermis (Figs. 1—3). This mass contains surprisingly few

nuclei, which appear to be "naked". The nuclei vary in their morphology. Characteristically they are rather small, smaller than the nuclei of the neoblasts. They are round, ovoid or irregular in shape. In most cases the chromatin is dense and the nuclei rather dark. In a few cases they are considerably lighter. Sometimes a nucleolus can be visualized. It is not possible with the light microscope to delimit the cytoplasm belonging to these nuclei. This is the reason why the parenchyma has often been described as a syncytium. The question about the syncytial nature will be discussed in a later section. The acidophilic mass is most compact in the head, tail and subepidermal region. It exhibits a more loose texture in the remaining regions of the body.

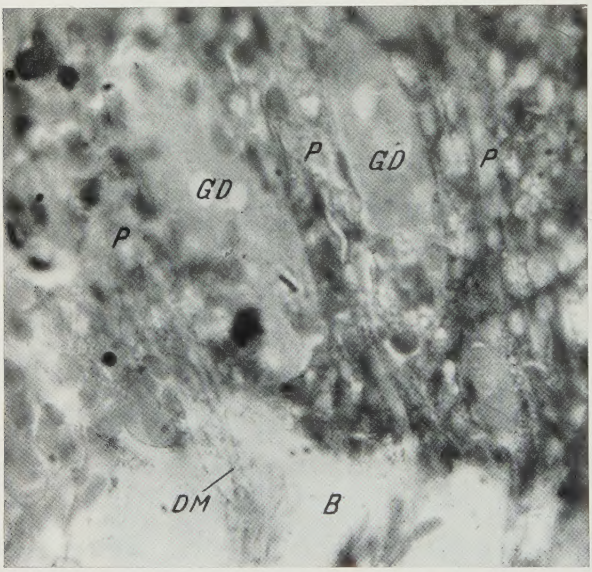

Fig. 3. Same as Fig. 2. Higher magnification (680 ×). The parenchyma (*P*) is very distinct against the faintly stained brain (*B*) and gastrodermis (*GD*). Note the dorso-ventral muscle (*DM*) going through the brain and with PAS-positive covering filamentous material

The question of the occurrence of fibrils in the connective tissue was examined by studying preparations stained with van Gieson, chlorantine fast red, aniline blue, elastin stain and silver impregnation methods. No collagen or elastic fibers could be detected. However, the subepidermal basement membrane was stained strongly with aniline blue (Fig. 1) and was impregnated by silver. It is moderately PAS-positive. Polarization optical examinations by TÖRÖK and RÖHLICH (1959) revealed an oriented organization parallel to the plane of the basement membrane. There was evidence of a fibrillar sheath of a similar nature investing the dorsoventral muscle fibers but this structure was much thinner than the basement membrane. A basement membrane covering the basal parts of the gastrodermal cells could not be observed in *Planaria vitta*, but there was some evidence at least for the existence of a slightly developed structure of this kind in *Dugesia tigrina*.

It was possible to obtain an improved demarcation of the parenchyma by using the PAS-reaction, since the parenchyma is intensely PAS-positive (Figs. 2, 3). The histochemical implications of this will be discussed in the section on the histochemistry of the parenchyma. Here it will merely be considered as a useful staining method. As a histological procedure it is still more useful when combined with picro — aniline blue (LILLIE's allochrome method) and gallocyanin. Because of the intensity of the staining, it is important to use thin sections. The reason for the superior demarcation is that the epidermis, gastrodermis, musculature, brain ganglia and nerve cords are only slightly PAS-positive, in contrast to the

surrounding parenchyma (Fig. 3). In sections stained with eosin, on the other hand, these structures are stained moderately, as is the parenchyma. Some basophilic gland cells are PAS-reactive and may give rise to confusion. This risk is minimized in allochrome-stained sections, which exhibit an excellent picture of the parenchyma, especially in frozen-dried planarians.

The PAS-positive strands were seen to penetrate between all organ-systems in the animal and were present almost everywhere, but were most highly concentrated in the spaces between the diverticulae of the gastrodermis. The strands also surround the brain ganglia and the nerve cords. As in the case of the preparations stained with acid dyes, the texture of the parenchyma stained with PAS appears to be more loose centrally. This is because the highest concentration of lipid droplets is found here and their dissolution during the preparation of the sections leaves empty spaces.

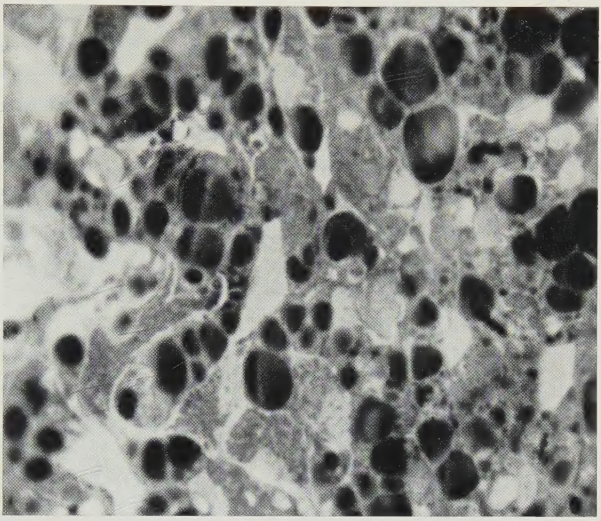

Fig. 4. 2 μ methacrylate section of osmium fixed *Planaria vitta*. Note the excellent tissue preservation and the many lipid droplets. The PAS-positive parenchyma (gray) is very distinct. The white areas represent cytoplasm and nuclei of cells other than the parenchyma cells and sometimes nuclei of parenchyma cells. PAS-reaction. 1160 ×

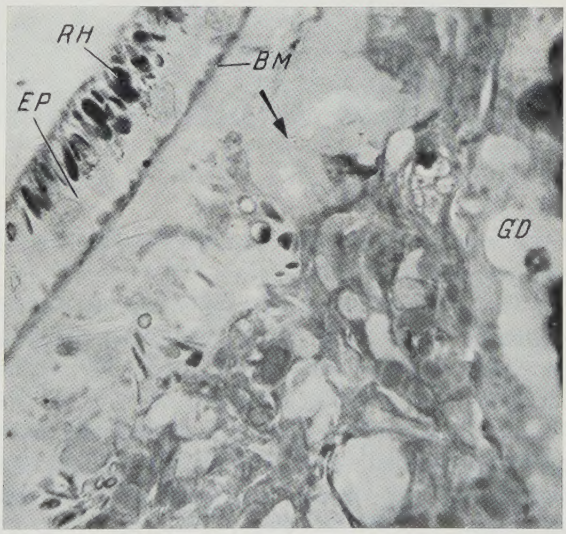

Fig. 5. Subepidermal region of *Planaria vitta* after osmium fixation and epon embedding. PAS and iron trioxyhematein. To the right is a divertivulum of the intestine (*GD*). The parenchyma is blackish gray. Note the well preserved excretion canal (arrow). 680 ×. (*EP* epidermis, *RH* rhabdite, *BM* basement membrane)

During the electron microscopical part of the present study, it was learned that the methods used for preparing tissues for electron microscopy were extremely valuable when applied to preparations destined for study with the light microscope. By using 0.5—3 μ sections embedded in methacrylate or Epon, it was for the first time found possible to obtain really meaningful histological pictures of the parenchyma.

The sections were treated according to the PAS-method and stained with iron trioxyhematein (HANSEN) without removal of the embedding medium. It was not found necessary to make any alterations in the PAS-procedure. However, even after one hour's staining with the nuclear stain, the nuclei were rather faint and were best observed under oil immersion. The stained sections were dried in air and mounted in glycerol or glycerol-gelatine. Occasionally epoxy resins were used as mounting media, but the contrast was not as high as obtained by using the other media. For phase contrast microscopy, the best con-

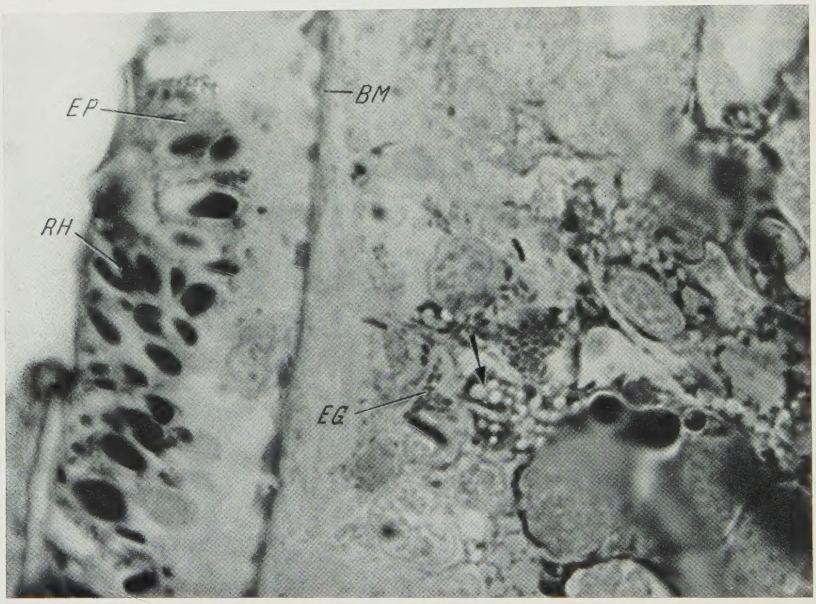

Fig. 6. As Fig. 5, higher magnification (1400 ×). Note the sharp demarcation of the parenchyma with lipid droplets (arrow). Note the section of an excretion canal in the upper right corner. Note the eosinophilic secretion granules (*EG*). (*RH* rhabdite, *EP* epidermis, *BM* basement membrane)

trast was obtained with methacrylate-embedded sections mounted in glycerol and rimmed with a resin.

The PAS-positive parenchymal strands were found to be exceedingly sharply delimited (Figs. 4—6). In thin sections, every small process was visible and the parenchyma could be followed in all its ramifications. The picture was very sharp, as most other tissue components, such as the nervous tissue, the intestine (except for PAS-positive inclusions and vacuoles), the neoblasts, etc., were completely unstained. For the first time, it was found possible to observe with the light microscope the excretory system in *Planaria vitta*. This was never achieved in ordinary paraffin sections. The lipid droplets were well preserved in Epon or methacrylate sections. The droplets were always surrounded by the intensely PAS-positive parenchyma. The droplets were largest and most numerous in the vicinity of the intestine. In the subepidermal region they were generally much smaller and in some places absent. But over all, the lipid droplets formed the most conspicuous part of the parenchyma in this species. It was found that the region between epidermis and gastrodermis was completely compact. No intercellular tissue spaces could be observed.

While the histological picture of the parenchyma thus was found to be very clear in *Planaria vitta*, a similar picture could not be obtained in *Dugesia tigrina*, even when prepared with the Epon technique. There was no doubt about the PAS-positive nature of the parenchyma in paraffin sections, but it could not be realized in the Epon sections. The reasons for this failure are not clear at the present. Part of the explanation may be sought in the longer fixation time employed for this species with a higher possibility for extraction. There may be a different chemical composition of the PAS-positive substances in the two species, perhaps involving a different susceptibility to the action of osmium tetroxide. It is possible that the pronounced difference in lipid content is in some way correlated with the PAS-reactivity in the surrounding parenchyma.

At this point it should be stressed that even by employing this technique, which is far superior to classical histological techniques, it was not found possible to give an unambiguous interpretation of the nature of the parenchyma. It was not possible to determine if ground substance was present, neither could it be decided if the parenchyma was of a syncytial nature.

In vivo observations

In order to study individual cells in planarian tissue, dissociation methods were adopted. It was hoped that isolation experiments might help in the interpretation of the nature of the parenchyma. The animals were cut into small pieces, placed in small microtubes and treated with 0.2 to 0.3% sodium chloride solution containing 0.05 to 0.5% crude trypsin. They were incubated either at room temperature or at 37° C for several hours. Before transfer to slides they were gently teased by needles. This treatment resulted in a fairly good general dissociation. The cells were viable for many hours. Even after 8 hours muscle cells were seen contracting and the ciliar tufts in the flame cells were active.

Many cell types were easily identified: epidermal cells, rhabdite forming cells, eosinophilic cells, pigment cells, phagocytes from the gastrodermis, muscle cells, flame cells and neoblasts.

However, it was found exceedingly difficult to identify the "fixed" parenchyma cells. From the study of light microscopical and electron microscopical sections it was inferred that these cells are large and highly polymorphic.

The few studies which have been done on planarian tissue culture (Murray 1927, 1931, Seilern-Aspang 1958, 1959) reveal the uncertainty with regard to these cells in the isolated state. Murray (1927) finds that it is difficult to identify the parenchyma cells (she does not distinguish between neoblasts and "fixed" parenchyma cells) from the point of view of either structure or function. She feels that the nature of the culture medium influences greatly the form of the parenchyma cells and finds in this an explanation for the conflicting reports on the histology of the parenchyma. She finds that the parenchyma cells are pseudopodial and of varying size, but smaller than the gastrodermal phagocytes.

In the present study similar cells were found in *Dugesia tigrina*. Under fortunate circumstances several larger cells were also observed, considerably larger than the phagocytes. The cytoplasm of these and the smaller cells was rather hyaline, often with granular or vacuolar areas. In a few cases they were provided with processes but usually the cells were rounded up or elongate with

a smooth surface. The nucleus was usually relatively small and provided with a nucleolus. The size of the cells was often astonishing, up to 50—75 μ in length. In *Planaria vitta* similar cells were found but they were usually loaded with lipid droplets and might be mistaken for phagocytes. In experiments done on *Planaria vitta* many isolated vesicles of varying size containing lipid droplets were generally seen, but without nuclei. This may point to the fact that the "fixed" parenchyma cells are fragile or that the vesicles may represent pinched-off cytoplasmic fragments of the "fixed" parenchyma cells (see the discussion).

Large cells comparable with the ones described, with a vacuolar cytoplasm and provided with processes, were observed by PRENANT (1922) after prolonged digestion of planarians with pepsin in hydrochloric acid. These he interpreted as "fixed" parenchyma cells.

It was found rather difficult to isolate these cells completely. In the experiments, sheets of partially dissociated tissue were often seen. It was thought that these sheets contained many of the "fixed" parenchyma cells.

In order to characterize the dissociated cells more closely, attempts were made to prepare them in fixed and stained smears. Practically all cell types were found to be fragile. The most gentle method tried consisted of careful smearing and fixation for a few minutes in osmium tetroxide vapour. After washing, the PAS-reaction and a nuclear stain was applied. The sheets were partially dissociated and showed a strong PAS-reaction in cells and cell fragments. However, this approach was not found to be especially valuable. No clear-cut results were obtained because too much damage was done to the cells during the preparation. Moreover, material was lost while the slides were immersed in the staining media.

In a previous paper (PEDERSEN 1959b) it was stated that neoblasts might be mistaken for "fixed" parenchyma cells in squash preparations. Continued study of the nature of the "fixed" parenchyma cells have, however, revealed that the two cell types are basically different as judged by both cytochemical and morphological criteria. The neoblasts have a larger nucleus than the "fixed" parenchyma cells and contain much less cytoplasm. Usually there is only a narrow cytoplasmic brim around the nucleus in the neoblasts while the "fixed" parenchyma cells are characterized by a large amount of hyaline and partially vacuolar cytoplasm. A drop of toluidine blue added to the isolated cells reveal the strongly basophilic neoblasts while the "fixed" parenchyma cells do not exhibit any basophilic properties. They are moderate acidophilic and can be followed by their PAS-positive cytoplasm.

Histochemical observations

In the following section the results described apply to *Planaria vitta* if not otherwise stated. The reactions relate to the acidophilic mass referred to in previous sections and which is interpreted to be the cytoplasm of the fixed parenchyma cells (see the electronmicroscopical section).

1. Methods for nucleic acids (Table 1). The Feulgen reaction for desoxyribonucleic acid was completely negative. The gallocyanin and pyronine staining methods showed only faint reactions. These reactions were only to a slight degree decreased after treatment with RNA-se. It may be concluded that there are only extremely small quantities of RNA present in these cells.

2. Methods for proteins and amino acids. The results are tabulated in Table 2a and 2b. It is seen that the reactions generally are faint to moderate and no particular amino acid side groups seem to dominate the chemical pattern. The observations concerning the nature of the acidophilia indicate a basic nature of the parenchyma. As the eosinophilia is completely abolished at p_H 11, the guanidine group of arginine is not important in producing the eosinophilia. This is in accordance with a virtually negative Sakaguchi reaction. As acetylation and nitrosation result in a considerable decrease of eosinophilia, amino end groups and amino groups in lysine may be responsible for the eosinophilia. WEISS et al.'s method for amino groups and the methods for basic proteins (naphthol yellow S and chromotrope 2 R) are also positive. However, in view of the results for demonstration of polysaccharides it must be kept in mind that amino groups in polysaccharides may exert their influence in the eosinophilic properties of the parenchyma.

It seems natural, however, to attribute the reactions observed to an unspecialized cytoplasmic matrix in the fixed parenchyma cells.

3. Methods for polysaccharides (see Table 3). As mentioned on several occasions, the parenchyma exhibits an intensely PAS-positive reaction. The PAS-

Table 1.
Reactions for nucleic acids

Method	Fixation	Result
Methyl green-pyronin	f	0 to +
	c	0 to +
Methyl green-pyronin + RNA-se	c	0 to +
Gallocyanin	c	0 to +
	f	0 to +
Feulgen	c	0
	f	0

c Carnoy, f buffered formalin, 0 negative, ± doubtful, + faint, ++ moderate, +++ intense.

Table 2b. *Reactions for eosinophilia*

Method	Fixation	Result
Eosin p_H 7	z	++
Eosin p_H 11	z	0
Eosin p_H 7 and acetylation	z	± to +
Eosin p_H 7 and nitrosation	z	± to +
Eosin p_H 7	acet-alc	+ to ++
Eosin p_H 7 and acetylation	acet-alc	0

z Zenker, acet-alc acetic acid-alcohol, 0 negative, ± doubtful, + faint, ++ moderate, +++ intense.

Table 2a. *Reactions for proteins and amino acids*

Method	Fixation	Result
Mercuric bromophenol-blue	z	+
Millon	f	+
Glenner's for tryptophan	f	0 to ±
Naphthol yellow S	z	++
	f	+
	h	++
Chromotrope 2R p_H 2.1	f	+
	z	±
Dihydroxy-dinaphthyl-disulfide (with and without reduction)	f	+
	z	+
	tca	+
Sakaguchi (McLEISH et al.)	z	0 to ±
	f	0 to ±
2-Hydroxy-3-napht-aldehyde	z	+
	acet-alc	+

f buffered formalin, z Zenker, h Helly, tca trichloro acetic acid-alcohol, acet-alc acetone-alcohol, 0 negative, ± doubtful, + faint, ++ moderate, +++ intense.

reaction was not decreased after diastase treatment, and it may be concluded that glycogen is not responsible for the reaction. Likewise, the reaction was not changed after treatment with hot pyridine for 24 hours and accordingly lipids can not be held responsible. In view of the rather faint reactions obtained in tests for protein and amino acids, it does not seem plausible that hydroxy-amino acids in proteins are the basis for the pronounced PAS-reactivity. Evidently this reaction must be attributed to polysaccharides.

Table 3. *Reactions for polysaccharides*

Method	Fixation	Result
PAS	f	+++
	z	+++
	h	+++
	os	+++
PAS and diastase treatment	f	+++
PAS and pyridine extraction	f	+++
Alcian blue	f	±
	z	0
Colloidal iron (MOWRY)	f	·
Metachromasia	f	0
	fresh-frozen	0
Sulfation and metachromasia	f	0
Sulfation and alcian blue	f	±

f buffered formalin, z Zenker, h Helly, os osmium tetroxide, 0 negative, ± doubtful, + faint, ++ moderate, +++ intense.

The metachromatic reaction was negative, both on frozen sections and on formalin fixed material in both species. No staining with alcian blue was observed and the colloidal iron methods gave negative to faint reaction. Thus, it seems safe to conclude that the polysaccharides are not of the acid type.

Several sulphation procedures were adopted in an effort to convert the presumed neutral polysaccharides into acid ones. These methods were only successful in the case of chlorosulfonic acid, but as all tissue components were rendered metachromatic it seems unwise to accept the result as evidence for a selective sulphation of neutral polysaccharides. Several neutral polysaccharides are notoriously difficult to demonstrate by sulphation techniques, e.g., glycogen (LEWIS and GRILLO 1959). The reason for the failure may be sought in extraction of the carbohydrates. In the present case another possibility may be considered. Relatively few hydroxyl groups may be present and the PAS-reaction be due to a preponderance of —C——C— groups. These groups may contribute to the

$$\begin{matrix} NH_2 & OH \end{matrix}$$

eosinophilia of the parenchyma. Furthermore, after sulphation, the spacings of the sulphate groups may be of a nature which does not give rise to metachromasia.

4. Enzymatic histochemistry (see Table 4). In a previous publication the histotechnical difficulties related to demonstration of enzymes in planarians were commented on (PEDERSEN 1959b). These difficulties were overcome in the present study by using cryostat methods. It was found possible to get excellent fresh-frozen sections of planarians by first quenching them in iso-pentane. Very good results were also obtained by a combination of formalin-calcium fixation and embedding in gum-acacia-sucrose with the cryostat technique. *Dugesia tigrina* was considerably better suited for certain of the enzymatic histochemical reactions, as the scarcity of lipid in the parenchyma greatly reduced confusion from

solubilization of lipid-soluble end products. Such a process was found to be the limiting factor in certain histochemical procedures in *Planaria vitta*. Accordingly, *Dugesia tigrina* was employed for the following reactions. In the parenchyma the succinic dehydrogenase and the cytochrome oxidase reactions were both found to be positive, but only very faintly and in scattered places. This perhaps corresponds to the scarcity of mitochondria in these cells (see the electron microscopic section). The alkaline phosphatase reaction was virtually negative. In animals starved for one week, the acid phosphatase activity revealed itself in a few scattered granules of the end product. This was the case after applying both the GOMORI method and BURSTONE'S method. Perhaps the reaction reflects the presence of lysosomes (phagosomes) in the parenchyma cells (see the electron microscopy section). A similar but more widespread reaction was observed in the case of non-specific esterase. The same explanation may be pertinent.

Table 4. *Reactions for enzymes*

Method	Fixation	Result
Alkaline phosphatase	fresh-frozen	0
	fca	0
Acid phosphatase (GOMORI) .	fresh-frozen	± to +
	fca	− to −
Acid phosphatase (BURSTONE)	fresh-frozen	± to +
	fca	± to +
Non-specific esterase	fresh-frozen	+
	fca	+
Succinic dehydrogenase . . .	fresh-frozen	± to −
Cytochrome oxidase	fresh-frozen	± to +

fca formalin-calcium, gum acacia-sucrose, frozen sections, 0 negative, ± doubtful, + faint, ++ moderate, +++ intense.

The enzymatic activity of the parenchyma is probably closely related to the problems of food transport and utilization in the animals and to the enzymatic activities of the phagocytes in the gastrodermis. A recent contribution to this field has been provided by ROSENBAUM and ROLON (1960). However, these authors did not investigate the transport of nutrients from the gastrodermis to the tissue of the planarians and the further fate of the food in the parenchyma. They found amino-peptidase activity in the parenchyma in starved animals and related this to a possible self-digestion of the parenchyma.

It is planned to pursue the enzymatic histochemistry of the parenchyma in further studies on the histophysiology of the parenchyma. It is felt that the parenchyma cells are so flexible and dynamic that a study of their enzymatic activity only gives real sense when correlated with their function. This is also the case, for example, with the phagocytes which display acid phosphatase activity only in certain phases of the intracellular digestion (ROSENBAUM and ROLON 1960) and the Minotian gland cells in the gastrodermis which exhibit reaction for non-specific esterase only in certain periods of their activity (PEDERSEN, unpublished).

Electron microscopic observations

As previously pointed out, even refined light microscopic examinations, using methods superior to conventional cytological technique, were not able to answer the fundamental questions about the nature of the parenchyma. The reasons for this failure were first fully realized after the introduction of electron microscopical methods to the problem. The tissue elements between the epidermal basement membrane and the gastrodermis were found to contain several cell types provided

with ramifying processes which form an extremely complex and elaborate pattern (Figs. 7, 8, 10, 11). A process of an eosinophilic gland cell may, for example, be so closely apposed to a rhabdite forming gland cell that it might be concluded from light microscopical observation that they were parts of a single cell. Numerous analogous cases are encountered and the frequent confusions or discrepancies in interpretation found in papers on planarian histology are easily understandable.

The fact that the light microscopical approach often may lead to wrong interpretations means, however, that in several cases it is necessary to evaluate electron micrographs without the valuable correlation which light microscopy offers. Accordingly, conclusions must be drawn cautiously.

In the preliminary stages of the present study, *Planaria vitta* was used for electron microscopy using methacrylate embedding. The preservation was not generally satisfactory but certain important facts emerged nonetheless. It seemed at once evident that the cells in the space between the epidermis and gastrodermis were very closely packed and many cell types were provided with intricately interdigitating processes. Because of this arrangement, practically no intercellular space was left. The significance of this finding will be discussed below. A striking feature was the occurrence of numerous broad or smaller processes of medium to low electron density. These processes usually contain extremely dense droplets of varying size, presumably of lipid nature. This surmise was corroborated by lipid staining methods. The processes are always invested with a 70 Å thick membrane. In many cases mitochondria are present. An endoplasmic reticulum could not be discerned and RNA-granules are only occasionally seen. This is also true of nuclei. The processes are most widely distributed between the ramifications of the gastrodermis and here the largest lipid droplets are also observed.

Epon embedding resulted in a great improvement in the preservation of structure for electron microscopy. However, because of the widespread occurrence of lipid droplets, the sectioning proved to be most difficult. Various degrees of hardness of the blocks and different infiltration times were tried, but still the sections were poor. Nonetheless, by using alternate thick (0.5 to 2 μ) sections treated with the PAS-procedure it was possible to demonstrate unequivocally that the pale processes seen in the electron micrographs correspond to the intensely PAS-positive material described previously. From this finding emerged the important fact that the PAS-positive material is present intracellularly and that the PAS-positive strands accordingly are cellular processes and not intercellular ground substance.

These processes are interpreted as constituting the cytoplasm of the fixed parenchyma cells. It was a most fortunate coincidence that these cells contained lipid droplets, for with this marker a more certain identification is possible. It should be mentioned, however, that the fixed parenchyma cells do not always contain lipid droplets. This is especially true in the subepidermal regions, and here the identification of isolated cell processes is accordingly difficult.

Although certain important facts emerged from the work done on *Planaria vitta*, the electron micrographs were still too poor in quality for a further evaluation of the cellular details and the cellular interrelationships. Accordingly, another species was examined: *Dugesia tigrina*. This species does not contain a significant amount of lipid in the parenchyma and sections of good quality could be obtained.

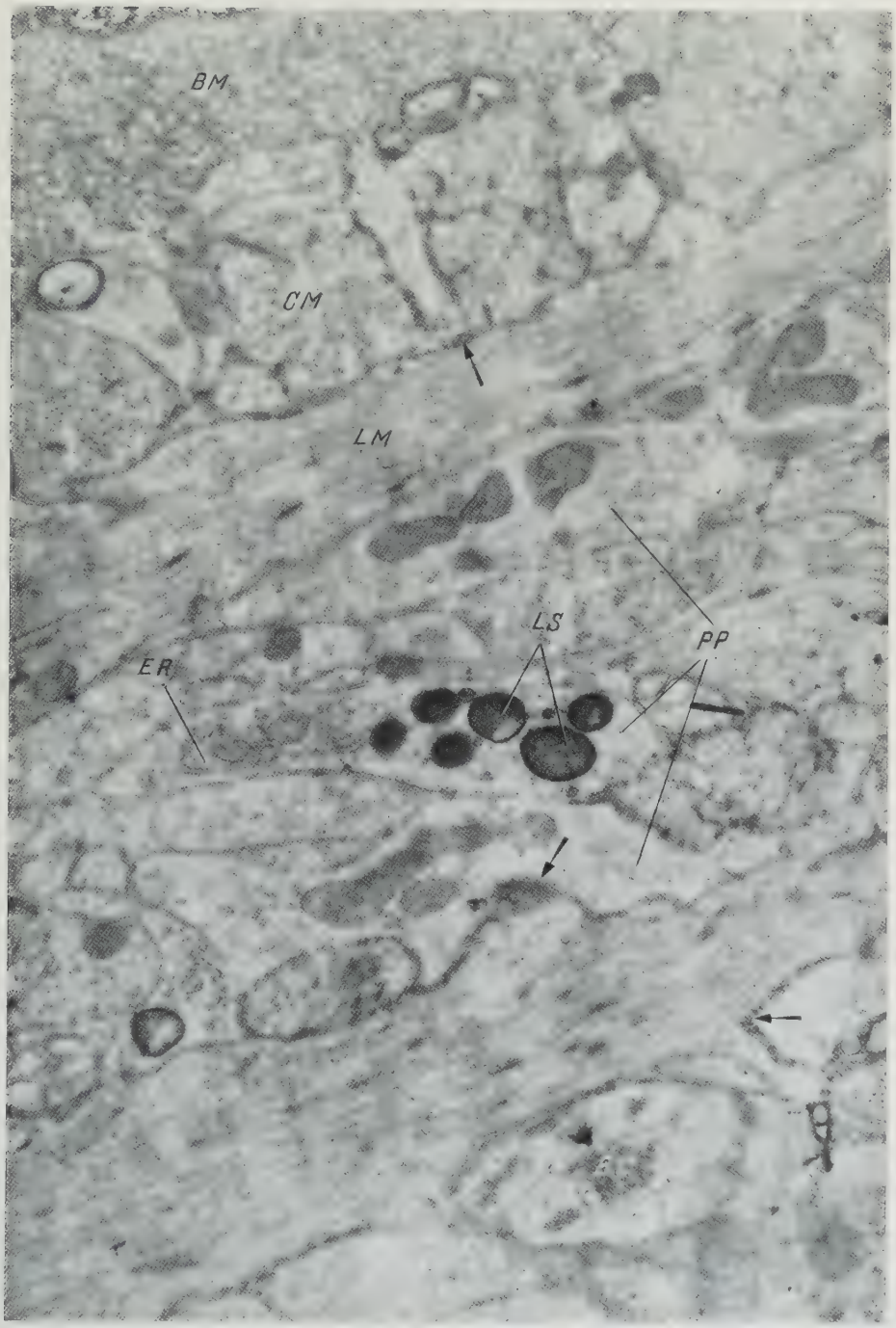

Fig. 7. A peripheral area *(Dugesia tigrina)*. At the extreme upper left is the epidermal basement membrane (*BM*). Under the basement membrane is situated the layer of circular muscle (*CM*) and then the longitudinal muscle layer (*LM*). Slightly below the center is a process of a fixed parenchyma cell (*PP*) containing rough surfaced endoplasmic reticulum (*ER*) and lysosomes (*LS*). Just below this another process from a fixed parenchyma cell with characteristic large mitochondria. Note the dense material in the intercellular space, especially around the muscle fibers (arrows). This is interpreted as filaments. 16,300 ×. (*BC* basophilic gland cell)

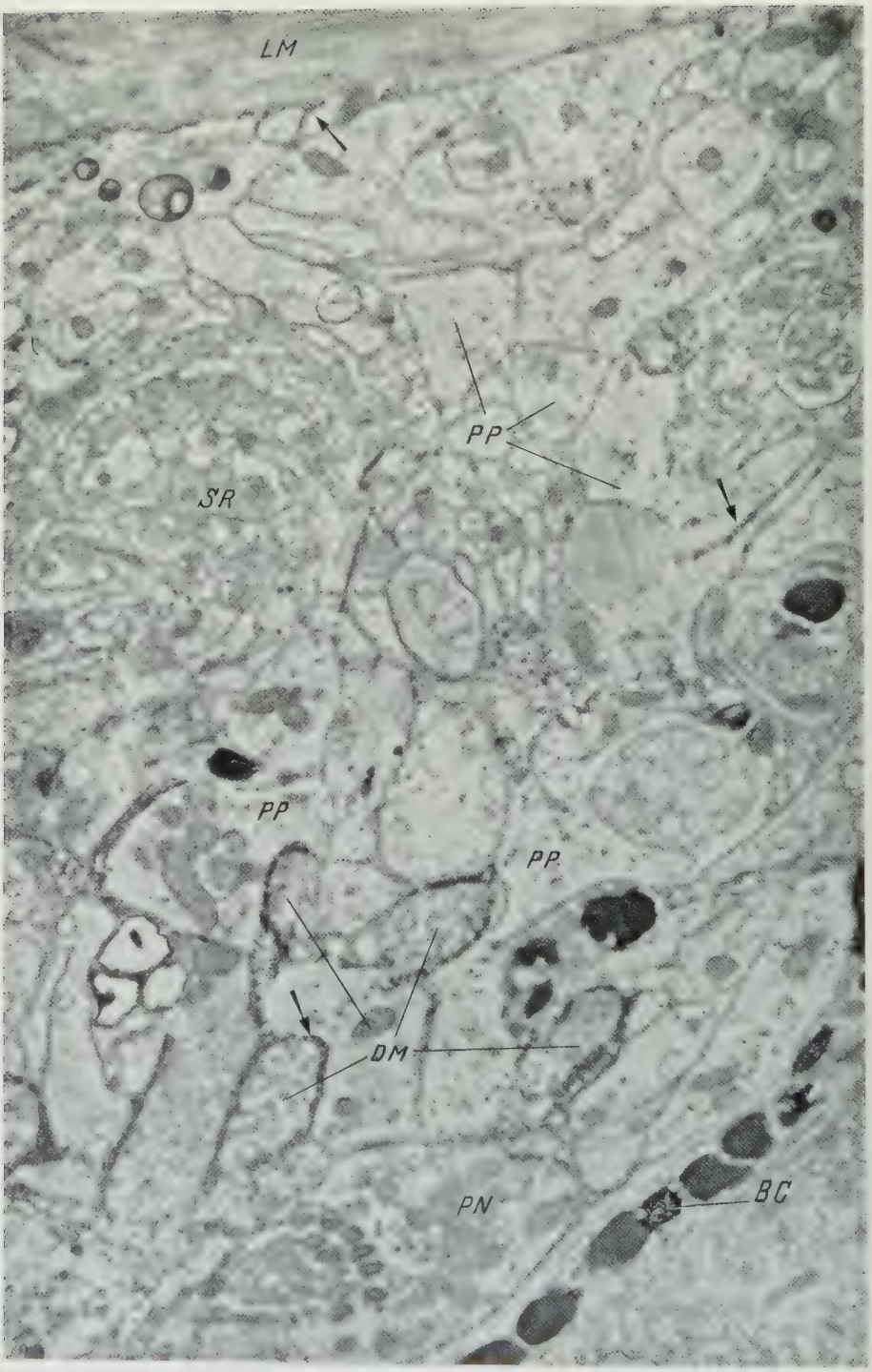

Fig. 8. A more central region in the parenchyma of *Dugesia tigrina*. At the top runs a longitudinal muscle cell (*LM*). A presumably synaptic region is found at the left (*SR*). There are several sections of dorso-ventral muscle cells especially in the lower left (*DM*). At the bottom is a section through a protonephridium (*PN*). Several secretion granules of the basophilic type are seen (*BC*). Note the dense intercellular material (arrows). 10,000 ×. (*PP* fixed parenchyma cell process)

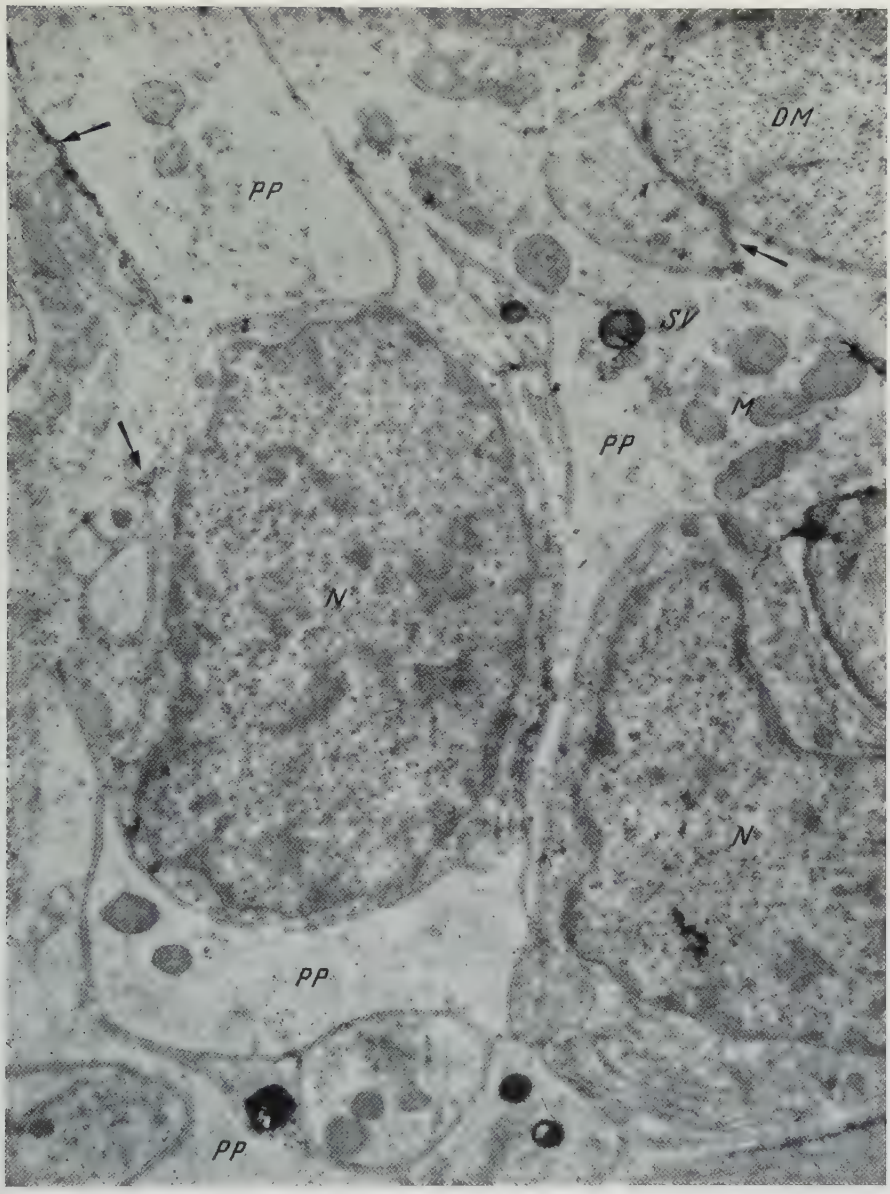

Fig. 9. Parenchyma of *Dugesia tigrina*. In the center a neoblast is seen partly surrounded by several fixed parenchyma cell processes (*PP*). Dense material occurs in the intercellular space (arrows). Several small vesicles (*SV*) are seen in the cytoplasm. Note the large mitochondria (*M*). 13,400 ×. (*DM* dorso-ventral muscle, *N* nucleus)

However, the identification of the fixed parenchyma cells is more difficult in this species because of the absence of lipid droplets. Also, as mentioned, the PAS-staining of adjacent thick sections was not successful with this species, and purely morphological criteria have to be relied on for distinction of cell types. Here the observations done on *Planaria vitta* proved to be of invaluable help.

Most of the sections examined were frontal or close to frontal. A few cross section were also studied. As the most important problem in this study was the

Fig. 10. Parenchyma of *Dugesia tigrina*. Two sections through nerves (*NV*) are seen and several broad processes of fixed parenchyma cells (*PP*). A process of a basophilic gland cell (*BC*) appears almost completely surrounded by the cytoplasm of a fixed parenchyma cell. Note that the dense intercellular material (cross sections through filaments) continue from the muscle (*DM*) and around the nerve. It is also evident in many other places (arrows). 8,100 ×. (*N* nucleus)

cellular relationships, high resolution work was only occasionally required and, accordingly, the majority of the photomicrographs were made at low to medium magnification.

As observed in *Planaria vitta*, the close packing of cells is a characteristic feature (Figs. 7 – 11). Because of the much better preservation of structure

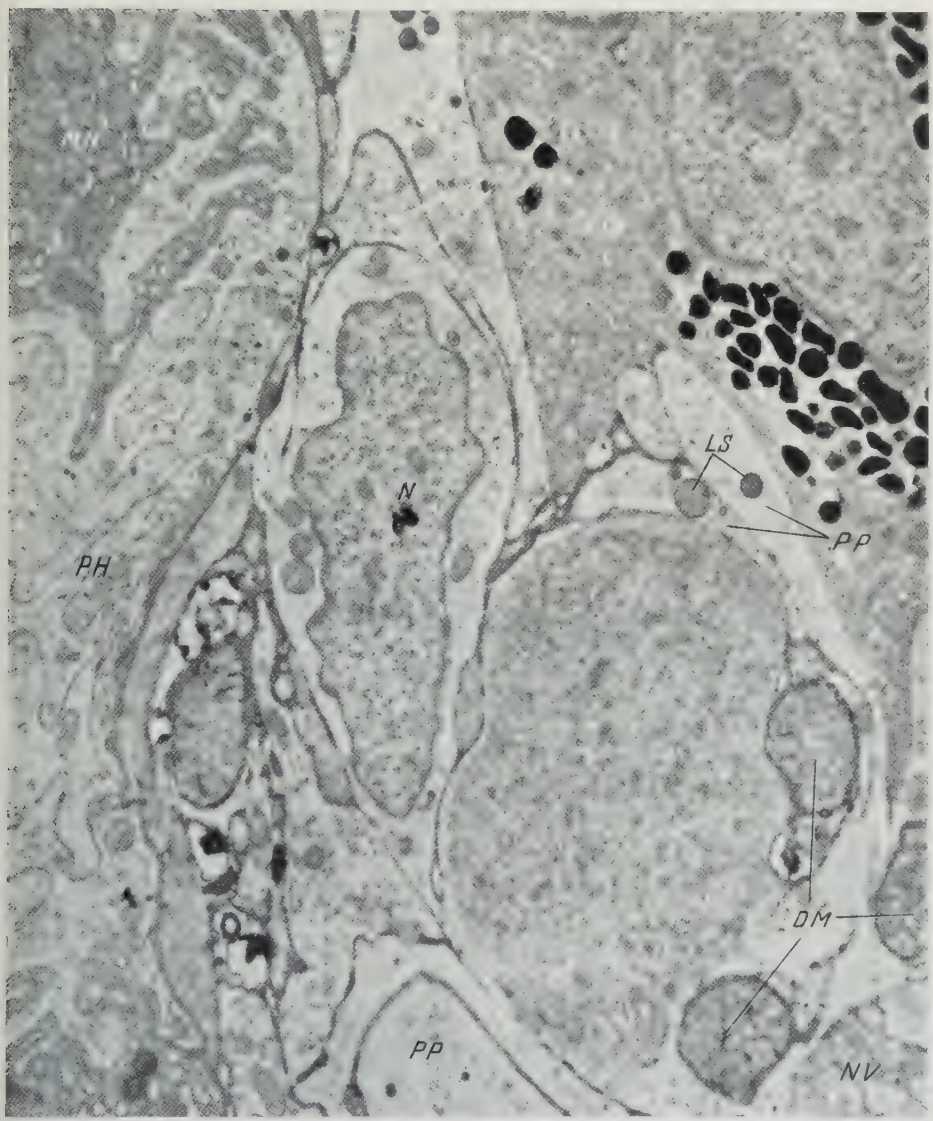

Fig. 11. Section of *Dugesia tigrina* at the base of the gastrodermis. The gastrodermis is at the left. The branching dark cell is a Minotian gland cell (*MN*) interdigitating with a phagocyte (*PH*). To the upper right a pigment cell is seen (*PC*). To the lower right is a section through nerve (*NV*). Several pale processes from the fixed parenchyma cells (*PP*) are seen, one containing a nucleus (*N*). Many small vesicles are seen in some of the processes. 8,850 ×. (*LS* lysosomes, *DM* dorso-ventral muscle)

in *Dugesia tigrina*, the interdigitating cell processes are better visualized. A large number of broad rounded processes exhibiting low electron density is a characteristic finding [Fig. 9—12 (*PP*)]. The close packing of cells leaves only a narrow intercellular space restricted to the compartments a few hundred Å wide between two apposing cell membranes. Occasionally this space is somewhat

widened, especially around the muscle cells (Fig. 13, 16). The shallow space is very often the site of medium dense material, which in many cases is organized as a thin line in the middle part of the space, leaving two broader regions of lower electron density against the cell membranes. The surprising finding of filamentous material in the narrow intercellular space will be commented on below.

Intercellular spaces larger than these compartments were not observed. The significance of this observation is that a real ground substance as observed in vertebrate connective tissue apparently does not exist in planarians.

Thus, the broad rounded processes are interpreted as cytoplasm of the fixed parenchyma cells. This interpretation is based partly on the observations on *Planaria vitta*, where these cells generally are "marked" with lipid droplets. In addition, it is based on the ubiquitous presence of these processes in regions where the "parenchyma" is known to occur from light microscopical studies.

This interpretation is felt to be valid in the case of the large, rounded processes. However, the finer ramifications cannot always be definitely identified. Here there is a possibility for confusion with the nervous tissue.

The present knowledge of the histology of the nervous system of triclads is in an unsatisfactory state. In most fresh water triclads, the nervous tissue is concentrated in the brain ganglia and in two well developed ventral nerve cords supplied with transverse commissures. Lateral branches are present. A loose submuscular plexus and, in some species, a subepidermal plexus is also a characteristic feature. The arrangement of the nerve fibers is quite well established and the uncertainty in identification mainly involves nerve cell bodies. This fact makes it difficult to identify nerve cells in electron micrographs. TÖRÖK and RÖHLICH (1959) have given the first description of the fine structure of nerve fibers in planarians: the sensory nerve fibers in the epidermis have a very pale cytoplasm and contain elongated mitochondria and structures which TÖRÖK and RÖHLICH identify as neurofilaments. The cristae of the mitochondria seem to be tubular rather than lamellar. The diameter of the filaments is about 120 to 200 Å. Besides these filaments, tubular structures with a greater diameter can be found running parallel to the fiber axis. These are presumably the smooth surfaced variety of the endoplasmic reticulum. WOOD (unpublished) has also observed tubular strands of the endoplasmic reticulum in nerve fibers in the brain region of *Dugesia tigrina* and he finds that these are centrally situated.

In the present study no emphasis has been laid on the structure of the nervous tissue and only a few observations were made on this subject. Transverse sections through whole nerves have been valuable for the identification and interpretation of this tissue. Purely longitudinal sections of neurons are seldom encountered and oblique sections are not easy to interpret. A single occurring thin axon or dendrite may be extremely difficult to identify. In cross sections, the occurrence of circular profiles perhaps corresponding to smooth surfaced endoplasmic reticulum was noted and these profiles are generally absent peripherially. The axoplasm exhibits a low electron density (Figs. 10, 11). Small mitochondria with lamellar cristae are observed. In the subepidermal region between the circular and the longitudinal muscle layer and just under the longitudinal layer, structures interpreted as synaptic regions are occasionally seen. Clusters of small uniform vesicles containing a medium dense substance and completely filling the cross

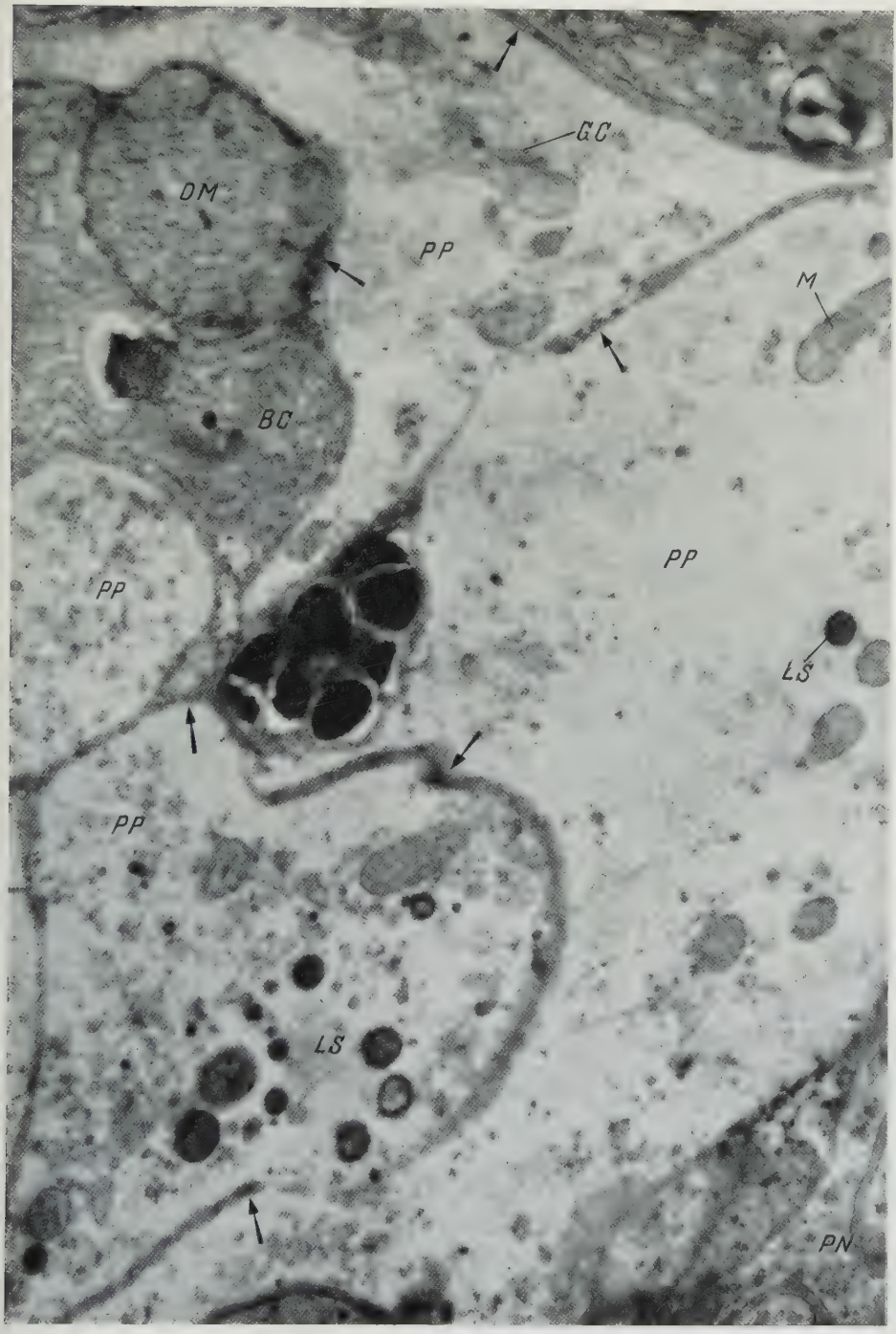

Fig. 12. Several processes of fixed parenchyma cells (*PP*) from the parenchyma of *Dugesia tigrina*. Note the material in the intercellular spaces (arrows). In one process membrane pairs are seen which are interpreted as Golgi material (*GC*). A dorso-ventral muscle cell (*DM*) is intimately in contact with a basophilic gland cell (*BC*). 16,200 ×. (*LS* lysosomes, *PN* protonephridium, *M* mitochondrion)

section of a fiber are a characteristic feature of many cell processes identified as nerve (Fig. 8, *SR*).

A further complication in the distinction between fixed parenchyma cells and nervous tissue is the uncertainty about the presence of neuroglia. The comparative histology of glia is only slightly worked out and especially the knowledge of the presence of glia in invertebrates is very scanty (GLEES 1955). In planarians practically nothing is known on this subject. This lack of knowledge poses the important question of whether glia cells are present in planarians and, if so, what the relationship might be to the fixed parenchyma cells. This question must be kept in mind in evaluating the validity of the interpretation of the parenchyma given in this paper. As will be mentioned in the discussion, there is a close morphological and perhaps also a functional similarity between the fixed parenchyma cells and the mammalian macroglia cells.

A description of the fine structure of the processes which are interpreted as belonging to the fixed parenchyma cells will now be presented. The cell membrane is 70 Å thick. Occasionally there is a suggestion of the existence of two peaks of density corresponding to the unit membrane structure as described by ROBERTSON (1959). The cell membrane is separated from adjacent cell membranes by a narrow intercellular space as described above. Desmosomes are not observed. It may be mentioned, however, that the septate desmosomes as described by WOOD (1959) are seen in three other locations in planarian tissues: between epidermal cells, between cells in the protonephridia and between phagocytes in the gastrodermis. The lack of desmosomes at the surfaces of the fixed parenchyma cells suggests a rather free mobility of this tissue.

The nucleus belonging to the fixed parenchyma cells is only rarely seen (Fig. 11). This is probably related to the large size of these cells. Also the light microscopical examinations reveal a surprising scarcity of nuclei in the acidophilic mass. The nucleus is generally irregular in shape in contrast to the nucleus of the neoblast, which generally is larger and of a more smooth contour. The chromatin is finely dispersed with a few coarser chromatin blocks present. The nucleus is bounded by a nuclear envelope consisting of two membranes bounding a medium dense interspace. A distinct, round nucleolus of granular appearance is observed. The polymorphic shape of the nucleus may be related to a presumed high degree of motility of these cells.

The cytoplasm varies in density. Usually it has a very pale, mottled grayish appearance (Figs. 12, 14, 15), occasionally a medium density but it is never higkly dense. Many processes have a nearly empty appearance (Fig. 9) but the density is always above that of the embedding material alone. The variable appearance of the processes is thought to be due to some extent to different functional states. The morphology of the cytoplasm probably also varies in different regions of the cell. The cytoplasm usually contains a fine, flocculent component (Fig. 12). Presumably this is the polysaccharide constituent which the fixed parenchyma cells contain in high concentration. The appearance is somewhat similar to the glycogen regions within liver cells. The cytoplasm often has a rather "washed out" appearance which could be due to part of the polysaccharide component being extracted during fixation and dehydration. Osmium tetroxide is generally not considered a good fixative for polysaccharides (BAKER 1958).

The rough surfaced variety of the endoplasmic reticulum is seen only rarely and is absent from most processes (Fig. 7). Free RNA-particles are more fre-

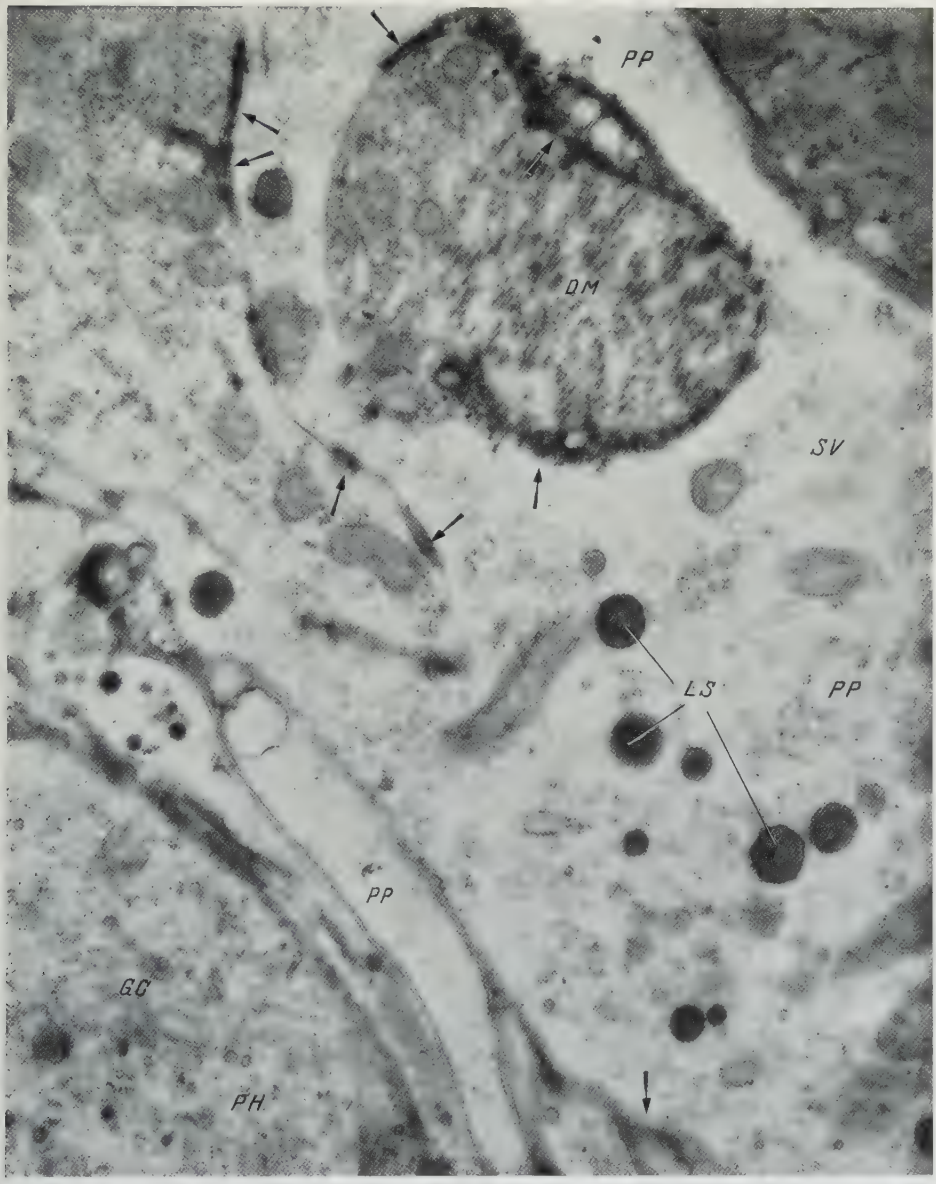

Fig. 13. Base of gastrodermis in *Dugesia tigrina*. To the lower left is part of a phagocyte (*PH*). Several processes of fixed parenchyma cells (*PP*) are in contact with this. Many irregular vesicles (*SV*) are seen and some lysosomes (*LS*). The filamentous nature of the intercellular material is evident in several places (arrows). 22,700 ×. (*GC* Golgi complex)

quently found. When present they appear clustered together in small groups. So few RNA-granules are present, however, that the very low basophilia due to RNA as observed in light microscopical preparations is satisfactorily explained.

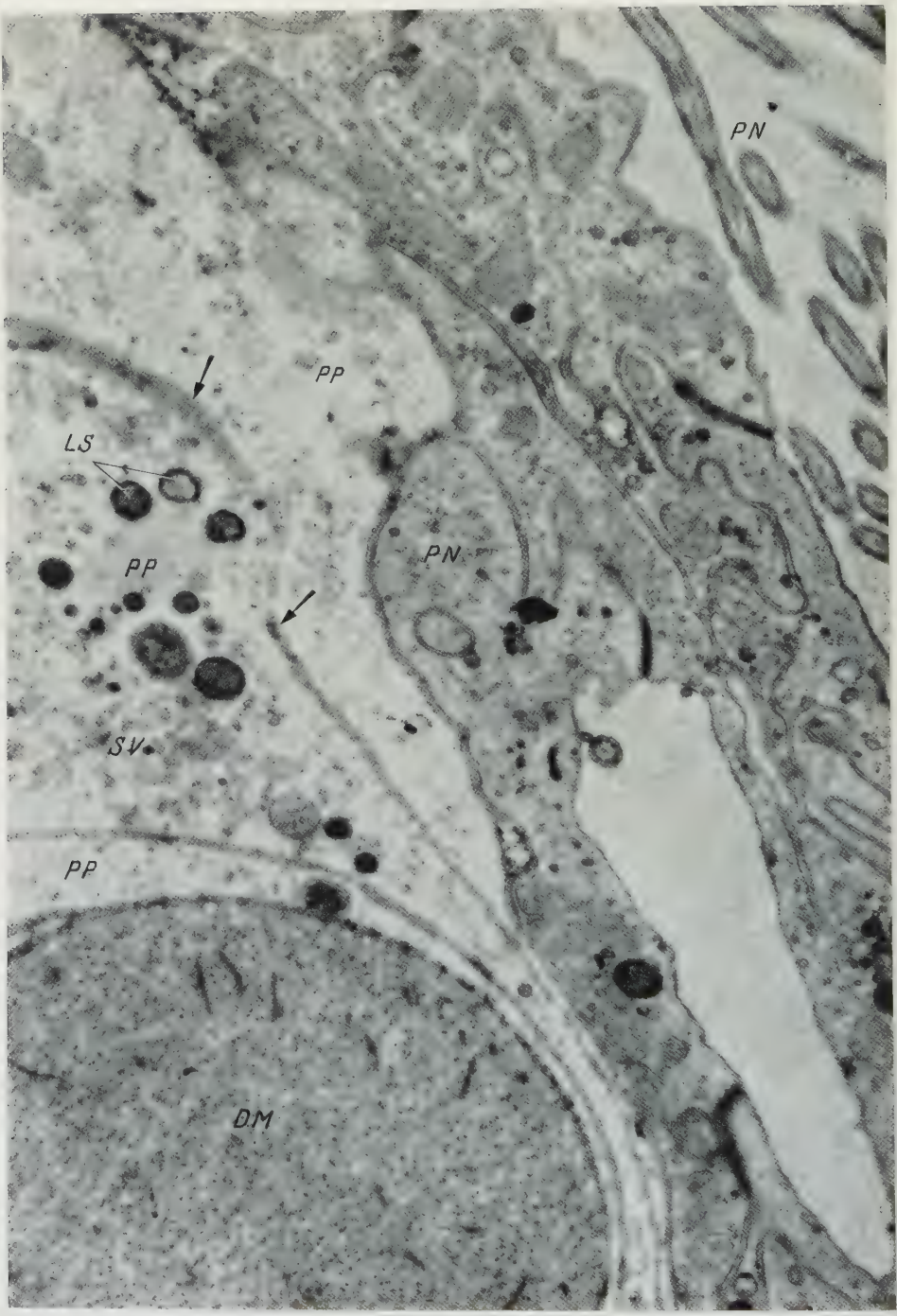

Fig. 14. A protonephridium in *Dugesia tigrina* (*PN*), closely invested by processes of fixed parenchyma cells (*PP*). To the lower left a dorso-ventral muscle fiber (*DM*) appears completely contained within the cytoplasm of a parenchyma cell. Lysosomes (*LS*) and small vesicles (*SV*) are seen. Material in intercellular space is evident (arrows). 16,600 ×

A Golgi complex is found occasionally. It consists predominantly of parallel placed closed membranes presumably forming flat sacks (Fig. 12, *GC*) but is rather inconspicuous.

In the majority of the cytoplasmic processes a few mitochondria are observed. Occasionally, however, rather large processes are found without these organelles. Evidently, the mitochondria are not evenly distributed and the number of mitochondria per cytoplasmic unit volume is probably low. In accordance with this, succinic dehydrogenase and cytochrome oxidase activities are low in the parenchyma. Mitochondria in planarian tissue cells are generally small and inconspicuous compared with mitochondria in most mammalian cells. In the fixed parenchyma cells, they are, for planarians, relatively large, about 0.3 μ in diameter. They display the general construction plan of mitochondria with two external membranes separating a less dense interspace and internal cristae, which are few in number and irregularly placed. The preferential orientation seems to be parallel to the long axis of the mitochondrion.

A general finding is a large number of irregular, circular profiles in the cytoplasm. These are interpreted as sections of vesicles (Figs. 12, 13). They vary somewhat in size and are generally a few hundred Å in diameter. Of course, a proportion of this variation is due to sectioning in different planes. Elongated profiles of the width of the vesicles are practically never observed. The thickness of the wall of the vesicles corresponds to the thickness of a cell membrane. The content of the vesicles exhibits a low to medium density similar to that of the cytoplasmic matrix. The number of vesicles vary much in different parts of the processes, perhaps a reflection of different functional states or of regional functional differences. The classification of these vesicles is difficult to establish. It might be argued that they belong to the smooth variety of the endoplasmic reticulum. On the other hand, they can scarcely be part of a reticulum if they are not connected, and there is no convincing evidence that this is the case. The vesicles differ in size and appearance from the synaptic vesicles.

An interesting outcome of this study is the demonstration of cytoplasmic structures presumably corresponding to "lysosomes". The question of the identification of these structures will be left for the discussion and here only their morphology will be described. In many processes characteristic highly dense structures are seen (Figs. 7, 13, 14, *LS*). They are variable in size, from 0.1 to 0.3 μ and are invested by a single membrane (occasionally two) displaying an intense density. The structures are usually round but may be ovoid in shape. The membrane is smooth or slightly wrinkled. The content of the structures is highly electron dense, but the density may vary in the same structure, to the extreme that occasionally in parts they may appear empty. The texture of the dense material varies from homogenous to granular.

Similar structures until now have only been observed in two other cell types in planarians, in the gastrodermal phagocytes and in some protonephridial cells. The possible functional implication of this distribution will be evaluated in the discussion.

In a few instances, similar but much larger structures are seen in the cytoplasm. These bodies resemble food vacuoles as observed in the gastrodermis.

The planarian parenchyma has often been described as being of a syncytial nature (HYMAN 1951). After the introduction of electron microscopy, many

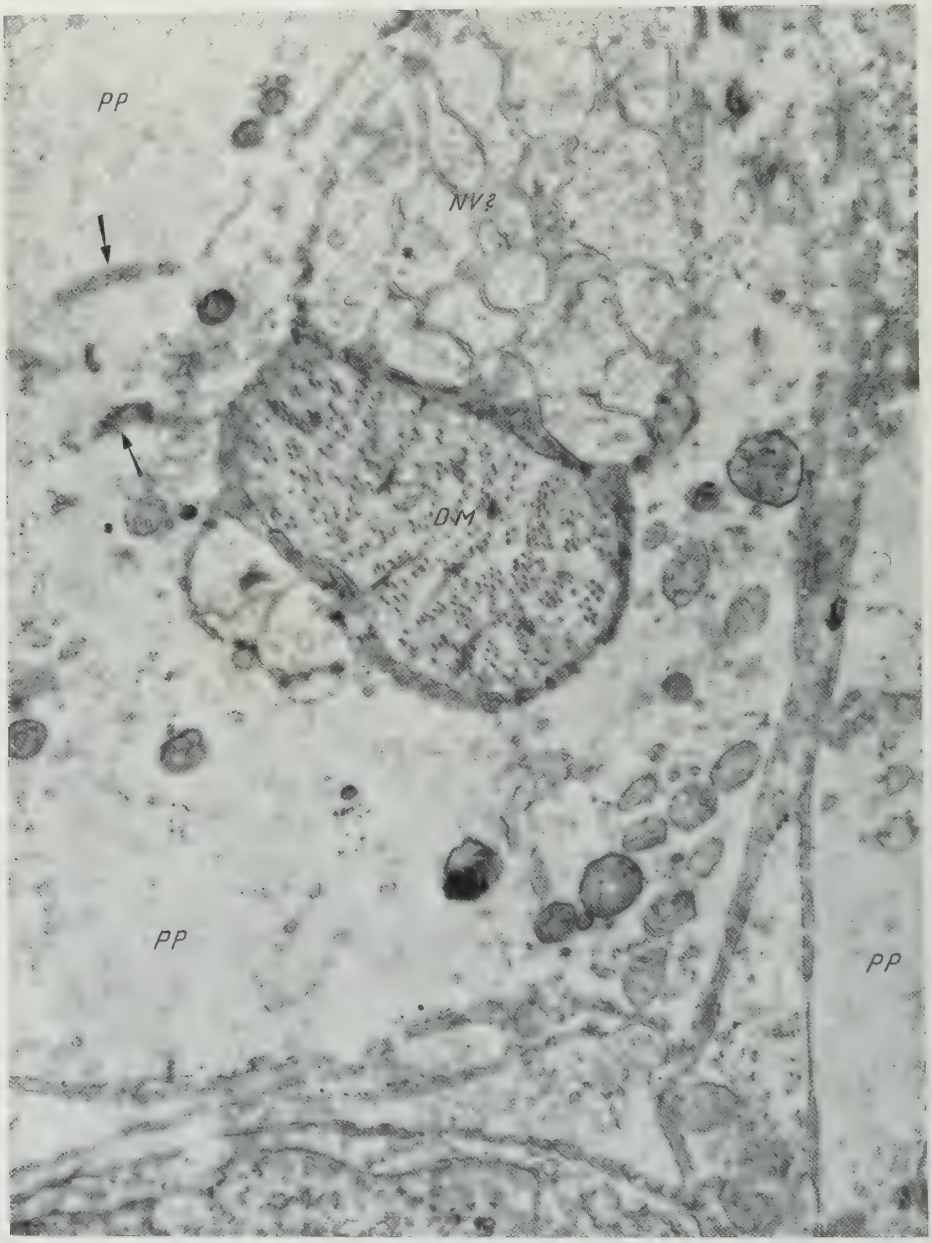

Fig. 15. A slightly oblique section of a dorso-ventral muscle cell (*DM*) perhaps in contact with nerve (*NV*) in the parenchyma of *Dugesia tigrina*. The filaments in the intercellular space are particularly evident. Oblique sections probably through the intercellular space between two fixed parenchyma cells (*PP*) are seen (arrows). Note the vesicles in the cytoplasm of the parenchyma cells 17,500 ×

structures previously claimed to be syncytial on the basis of light microscopical studies were found to be truly cellular. In these electron microscopical studies

of planarians, cell membranes could always be distinctly delimited. Further comments on this point will be postponed for the discussion.

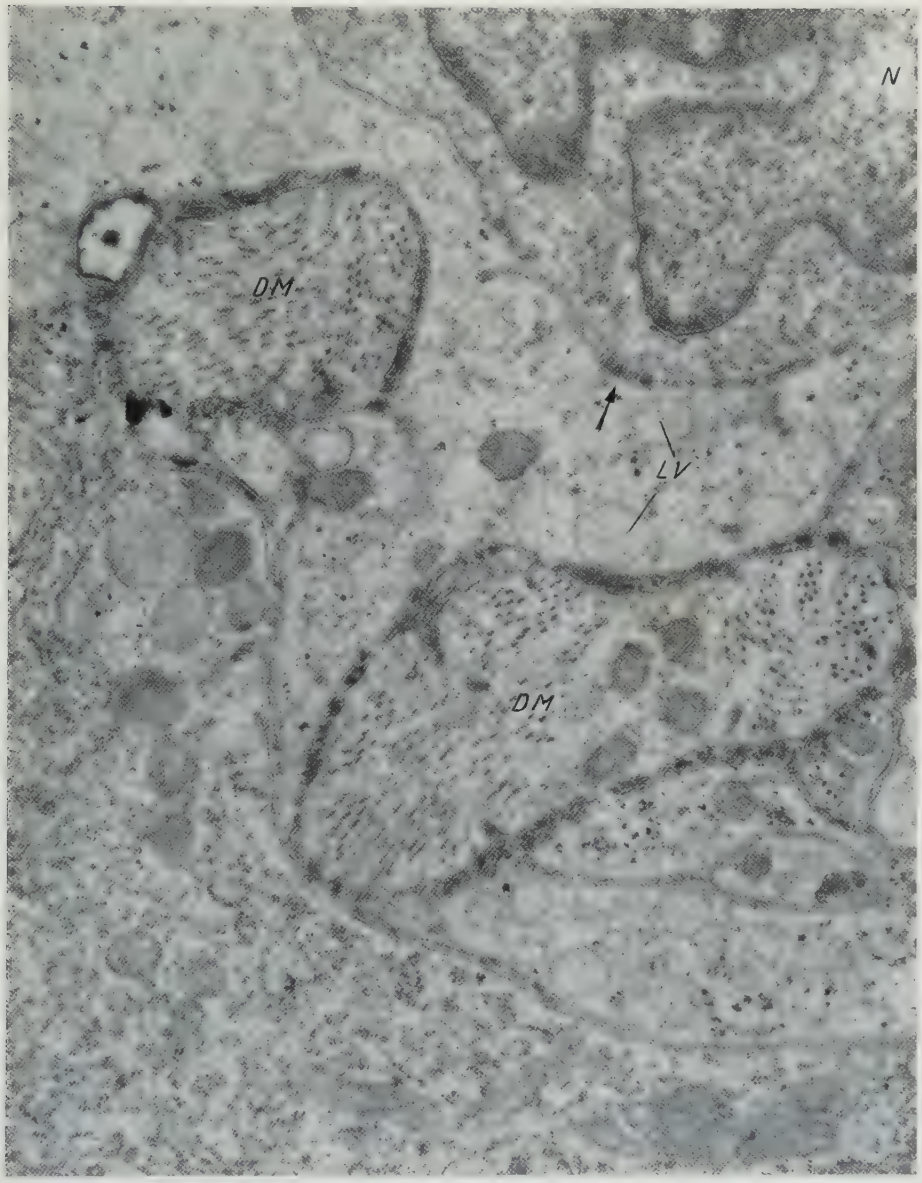

Fig. 16. Two dorso-ventral muscle cells in *Dugesia tigrina* (*DM*). The process going from the upper left to the lower right may have its origin from a fixed parenchyma cell and the vesicles (*LV*) may be final stages of lysosomes. Note the filaments in the space between the cell to the upper right and the central one (arrow). 24,100 ×

The cellular components of the planarian connective tissue have been described. It was pointed out that, in general, ground substance is absent with the exception perhaps of a slight amount of material occupying the extremely narrow space

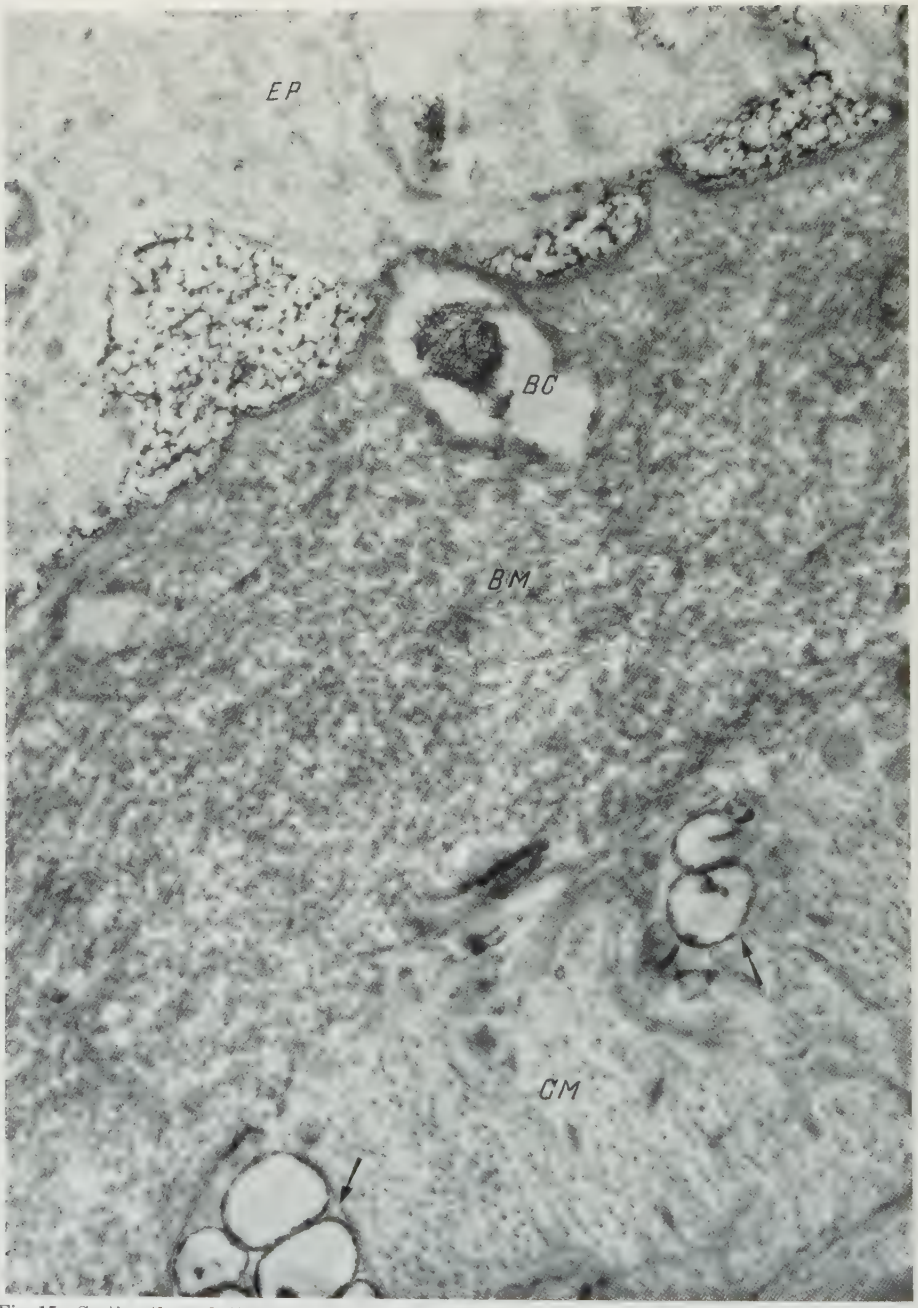

Fig. 17. Section through the subepidermal basement membrane (*BM*) of *Dugesia tigrina*. Epidermal cells are in the upper left corner (*EP*). Dense, probably artifactual, material lies between the basement membrane and the epidermis. The structures indicated by arrows are widespread under the basement membrane, but never above it. Their nature is unknown. A process of a basophilic gland cell (*BC*) penetrating the basement membrane is seen. 25,200 ×. (*CM* circular muscle)

between two apposing cell membranes. The third constituent of planarian connective tissue, the filamentous component, will now be considered.

From light microscopical work, it was believed that fibrils could be only a very minor constituent of the connective tissue. This is, of course, a consequence of the nearly complete absence of intercellular space and ground substance. However, in several localities distinct filamentous material is present, most prominent in the subepidermal basement membrane.

The subepidermal basement membrane is a highly developed structure (Fig. 17). Only a few of its interesting features will be commented on in this paper. The basement membran is very thick, about 1 μ, and it is perforated in many places by cellular processes. The peripheral surface facing the epidermal cells is smooth and is delineated by a dense limiting layer (Fig. 17). A space is often present between the basal parts of the epidermal cells and the basement membrane and this is occupied by an extremely dense network (Fig. 17). This is perhaps a fixation artifact produced by precipitation of protein or lipid material. The part of the basement membrane facing the subepidermal circular muscle layer is irregular and of highly elaborate structure. This will be analyzed at another occasion in relation to a study on the structure of the planarian musculature. The major part of the basement membrane is occupied by filaments about 75 to 100 Å in diameter. The filaments are closely packed and found to run in all directions but predominantly parallel to the plane of the basement membrane. This orientation was also suggested from the polarized light studies of Török and Röhlich (1959). These authors also described filaments 100 Å in diameter in the species *Dugesia lugubris*. Their finding that the filaments consist of a dense outer zone surrounding a center of less density could not be corroborated in this study. Often, however, two filaments are running parallel to each other and this could give rise to the above interpretation. The filaments often exhibit a beaded appearance but a real periodicity was not discerned at the resolution attained. The filaments are embedded in a ground substance of low to medium electron density. Because of the close packing of the filaments, the amount of ground substance is slight.

The intercellular space between muscle cells and neighboring cells is often wider than the usual distance between two apposing cell membranes. In this space filamentous structures are generally seen (Figs. 13, 15, 16). They display an intense density, sometimes higher than that of the myofilaments. These filaments have a smaller diameter than the myofilaments (about 75 to 100 Å) and they are oriented parallel to the direction of the muscle fiber. They have a slightly beaded appearance.

It was often observed that the narrowing intercellular space continuous with the space around the muscle cell also contained similar filaments. For example this is true in the intercellular space surrounding nerves (Figs. 10, 15). Between other cell types, a less frequent occurrence of similar filaments was observed (Figs. 7, 12—14). In several instances oblique sections revealed filaments in the space between fixed parenchyma cells (Fig. 18). Also around the gastrodermal cells similar filaments are frequently found.

The dimensions of the filaments are similar to those of the basement membrane and the two kinds of filaments might be identical. In support of this hypothesis it might be mentioned that there often seems to be a continuity between the subepidermal basement membrane and the intercellular space around the outer

circular muscle cells (Figs. 7, 17). Perhaps the filaments together represent a kind of primitive reticular sheath supporting the animal.

From a comparative point of view, the presence of extracellular filaments in planarians is most interesting. Although the intercellular space is so slightly

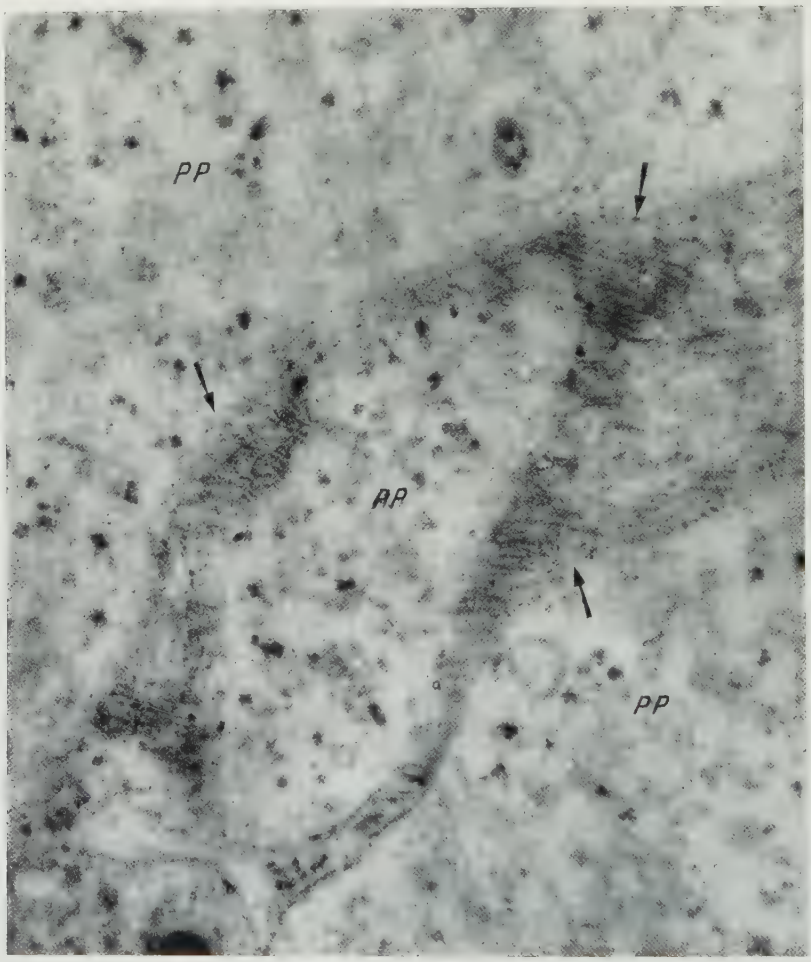

Fig. 18. Oblique section through fixed parenchyma cell processes in *Dugesia tigrina*. Note the filaments in the interspace between adjacent parenchyma cell processes (*PP*). 36,700 ×

developed as to be restricted to the narrow space between two apposing cell membranes and the ground substance, if present at all, is extremely sparse, there is, nonetheless, a capacity for formation of filaments.

This raises the important question of the origin and formation of these filaments. Nothing definite can yet be said about this intriguing problem. No filamentous structures were ever observed inside the neoblasts or the fixed parenchyma cells in intact planarians. However, in regenerating planarians the first sign of repair of the subepidermal basement membrane is the occurrence of small PAS-positive granules associated with the old intact basement membrane. The

origin of these granules is unknown. In insects, which have very sparsely developed connective tissue, WIGGLESWORTH (1956) has demonstrated that basement membranes have their origin from PAS-positive granules, and that these granules are produced by a certain kind of blood cells, haemocytes.

The question as to the origin of the connective tissue filaments in planarians will be dealt with in a following study on the differentiation processes in the regenerating head blastema. Preliminary studies on 5 day blastemas have revealed the presence of filaments in the interspace between muscle cells and neighboring cells.

A closer study of the filaments with higher resolution and a study of their relationship to the connective tissue cells will also be postponed to that occasion. However, there are already suggestions that the filaments predominantly occur in intercellular spaces where a fixed parenchyma cell is present on at least one side.

Discussion

The foregoing observations have clarified many concepts about the nature of planarian connective tissue and a highly interesting picture has emerged. The connective tissue is primarily cellular and the majority of the cells or at least the main mass of cytoplasm is constituted by the fixed parenchyma cells. The second cellular component consists of the neoblasts.

The cells are very closely packed and only the narrow interspace between two apposing cell membranes is left as intercellular space. It is possible that part of the medium electron dense material present in this space may represent ground substance. The cells do not seem to form syncytia.

In a few cases the intercellular space is slightly widened, especially around muscle fibers. Here definite filaments are observed. A frequent appearance of filaments in the narrow intercellular space between other cell types is also observed. The major filamentous component of the connective tissue, however, is found in the subepidermal basement membrane.

How does this picture compare with the classical descriptions of the planarian parenchyma? Two main hypotheses can be said to dominate the literature (PRENANT 1922). One hypothesis postulates that the parenchyma is formed by large vacuolar cells and the spaces observed are of intracellular nature. The other hypothesis considers the spaces extracellular. Some of the authors do not comment further on these spaces (CHICHKOFF 1892, MICOLETZKY 1907), while others find that the intercellular spaces contain ground substance or "perivisceral fluid" (UDE 1908, SCHNEIDER 1908, LANG 1912).

Another question which especially dominated the early literature pertained to the presence of connective tissue fibers. However, it seems certain that the dorso-ventral muscle fibers often were included in the description of the parenchyma as commented on by CHICHKOFF (1892), in this way giving a false impression of the presence of connective tissue fibers. In general, the existence of such fibers is denied, except in the case of the subepidermal basement membrane (IIJIMA 1884, CHICHKOFF 1892, UDE 1908, SCHNEIDER 1908, PRENANT 1922). For a further discussion, see below.

A penetrating study of the planarian parenchyma (and the parenchyma of *Platyhelminthes* in general) was carried out by PRENANT (1922). He employed

most histotechnical procedures available at that time including isolation of cells with pepsin. He found that the ground substance is reduced in amount in triclads and that free cells and large "fixed" cells are present. He also included the basophilic and eosinophilic gland cells as well as rhabdite forming cells as true parenchymal elements. He interpreted the fixed cells as forming a reticulum and comments that the cells are difficult to distinguish and that they often are obscured by other elements even in thin sections. He was able to isolate the fixed cells and demonstrate their large size, vacuoles and many processes. He noted polymorphism in the cells and felt that they vary in form according to age. He described a reduction in cytoplasm and a degeneration of the nucleus in older cells. PRENANT identified lipid droplets and believed that the cells are actively secreting. He found a very close relationship between ground substance, which has "reticulin like" properties and the fixed cells and interpreted the ground substance as cell processes which have undergone a "chemical transformation".

A similar interpretation of the ground substance was given by BÖHMIG (1891) in the case of rhabdocoel turbellarians. He stated this in the following way: „Das Gerüstwerk des Parenchym ist die Summe der Zellgerüste, die körnige Zwischensubstanz wird aus dem Zellsaft der einzelnen Zellen gebildet." BÖHMIG felt that the limits between the cells disappear during development and that intercellular spaces are formed.

IIJIMA (1884) found that also in triclads a syncytium is formed from original individual cells.

From these and many other classical studies emerged the picture which has dominated the concepts of the connective tissue in planarians. This has been summarized by HYMAN (1951). She considers the mesenchyme as "a fixed net-like syncytium and of free cells that wander about through the syncytium (PRENANT 1922). The syncytium is nucleated, often has a fibrillar appearance, and contains many fluid-filled interstices."

It is evident, however, that there are strongly opposed opinions on the structure of the parenchyma. It should be stressed, moreover, that generalisations are dangerous in this field. According to PRENANT (1922) many variations are possible in the general scheme of the parenchymal construction among the *Platyhelminthes*. The present investigation confirms this variation. In *Planaria vitta* many lipid droplets are present in the fixed parenchyma cells, while the same cells in *Dugesia tigrina* are practically devoid of these. Fundamentally, however, the construction of the parenchyma in these two species is quite similar.

The findings in the present paper are in accordance with the minority of authors who found that ground substance is absent and that the main component of the parenchyma consists of large cells provided with many processes. Even if these authors found intercellular spaces, they did not describe the presence of ground substance (CHICHKOFF 1892, MICOLETZKY 1907). The spaces are probably artifacts produced by the histological processing.

The findings are also partially consistent with the masterly interpretation of PRENANT (1922). His description of a reticulin-like substance has its counterpart in the strong PAS-positive cytoplasm of the fixed parenchyma cells. Also the isolation experiments done so far corroborate PRENANT's findings.

The syncytial nature of the parenchyma has been questioned by MURRAY (1931) and REISINGER (1959) who examined living tissues. MURRAY found that by cultivating pieces of parenchyma, cells roll out and are perfectly distinct from each other. REISINGER commented that the cellular nature of the triclad body tissues cannot be held in doubt, but in certain cases of cellular activity a reversible syncytial transformation may take place, e.g. in the gastrodermal phagocytes. He also found that in the highly interesting "Restitutionskörper" or "Sphärien" which develop after mechanical dissociation of planarians, a syncytium of connective tissue cells is formed. Since he used only light microscopy, this interpretation is, however open to question. As commented on by BARGMANN and KNOOP (1959), electron microscopical studies have revealed the true cellular nature of many structures previously held to be syncytial. These authors also discussed the definition of the terms "syncytium" and "plasmodium" and found that a confusion of these terms is often observed in the literature. By "syncytium" they understand "einen Zusammenschluß von Zellindividuen unter Aufgabe ihrer Grenzmembran" and by "plasmodium": "eine große vielkernige Zytoplasmamasse, die aus einer Zelle unter wiederholter Kernteilung bei ausbleibender Zytoplasmateilung entstanden ist." According to these definitions, the occurrence of "intercellular bridges" as seen between cells in mammalian testis or between ectoderm cells in *Hydra* (FAWCETT, ITO and SLAUTTERBACH 1959) do not constitute a syncytial arrangement of the cells involved. BARGMANN and KNOOP considered these as special of plasmodia. These authors conclude that they agree with CAESAR, EDWARDS and RUSKA (1957) who believe that syncytia do not occur in vertebrates.

The present investigation demonstrates that an invertebrate tissue hitherto held to be syncytial by many workers is also not truly syncytial. Observations on the planarian gastrodermis, also reported to be syncytial by some authors, have indicated that intricate cellular relationships occur, but a syncytium does not occur (PEDERSEN 1961). REISINGER's claim that the "Sphärien" are syncytial should be corroborated by electron microscopical observations.

It would be of interest to be able to relate the structure of the planarian connective tissue to connective tissue structures in other groups in the phylum Platyhelminthes and to other animal phyla. However, this comparative approach would be an immense undertaking and perhaps not very illuminating since very little work has been done in this field with the application of modern cytological methods. It seems evident from the present work that only such methods are able to give reliable data on certain of the basic problems encountered in this difficult field of animal histology. Some aspects of the comparative histology of connective tissue has been reviewed in PRENANT, BOUIN, MAILLARD (1904), SCHNEIDER (1908), DAHLGREN and KEPNER (1930), RUDALL (1955) and ANDREW (1959).

As planarians belong to a phylum where a "true" mesoderm (entomesoderm) first is believed to occur, it seems pertinent once more to stress the peculiar fact that the connective tissue is predominantly cellular and that ground substance is nearly completely absent because of the close packing of cells. This arrangement generally only leaves a narrow intercellular space between two apposing cell membranes. Most interestingly, a frequent occurrence of filaments is observed

in this space. Perhaps this arrangement is one of the most primitive expressions of a connective tissue in the three-layered animals.

It is difficult and perhaps arbitrary to define what is understood by more or less "primitive" connective tissue. If, however, the mammalian connective tissue is chosen as an example of a highly developed one, then the criteria will depend on the presence of cells, above all fibroblasts and, most characteristic, an abundant occurrence of ground substance and highly organized filaments of the collagen type (reticulin and collagen) exhibiting a characteristic x-ray diffraction pattern and a distinct periodicity (640 Å). Chemically, the protein of the filaments is characterized by the unique presence of the amino acid hydroxy-proline. Generally speaking, the extracellular components are dominating in mammalian connective tissue, both morphologically and functionally.

Judged by these criteria, the planarian connective tissue is very primitive indeed, perhaps even more primitive than the mesogloea of the *Porifera*. Sponges often contain a large amount of ground substance very similar to the mammalian ground substance (GROSS et al. 1956) and the characteristic "spongin" shows close similarity to mammalian collagen (RUDALL 1955, GROSS et al. 1956). Among the coelenterates, the hydrozoan medusae, *Schyphozoa* and *Anthozoa*, have a high collagen content in their mesogloea (RUDALL 1955). In *Hydra*, the sparse mesogloea contains filaments similar to the filaments found in planarian connective tissue (WOOD, in preparation), but *Hydra* generally seems to have a less developed connective tissue than planarians.

It is generally agreed that the parenchyma has important functions besides the primary supportive one (BÖHMIG 1891, PRENANT 1922, BRESSLAU 1928–1933, HYMAN 1951). The fixed parenchyma cells are eminently suited for support. Even from the static picture presented by electron micrographs, one gets the impression that these cells are very flexible and this is corroborated by observations on the living cells. In cultures, gland cells are often seen to pinch off bags of secretion granules invested by a cell membrane and occasionally a similar process has been observed in the fixed parenchyma cells. Whether this interesting phenomenon takes place in the animal itself is unknown. The probable absence of desmosomes is a further indication of the mobile nature of the fixed parenchyma cells.

As pointed out by JONES (1957), the mammalian connective tissue is a major food depot, water depot and electrolyte depot. The high lipid content in the fixed parenchyma cells in *Planaria vitta* suggests a depot function for these cells also. Whether the high content of PAS-positive components, presumably polysaccharides, has a nutritive significance has not been determined. It may function as a matrix for exchange of water and electrolytes in a manner similar to the ground substance in vertebrate connective tissue. The presence of a cell membrane around the polysaccharide matrix would not make much difference to such an exchange, as these cells possibly are engaged in pinocytotic activity as suggested by the presence of many small cytoplasmic vesicles.

The general relationship of connective tissue to repair and regenerative processes in vertebrates is also highly characteristic for planarians. The neoblasts are the cells responsible for the regenerative capacity. Destruction of these cells with x-ray radiation (DUBOIS 1949) or the radiomimetic substance triethylene

melamine (Pedersen 1958) results in a complete repression of regeneration. Whether the fixed parenchyma cells play any role in regeneration is unknown. In the classical literature on planarian regeneration a much debated question concerns the relationship between the neoblasts and the fixed parenchyma cells (Lang 1912, Steinmann 1912—1917, Prenant 1922, Brøndsted 1955). In the author's opinion there is no doubt about the fact that these two cell types are completely different. This is borne out by morphological, histochemical and functional considerations. However, during regeneration the totipotent neoblasts probably differentiate into fixed parenchyma cells. But it is thought highly improbable that fixed parenchyma cells ever are de-differentiated to neoblasts. Mitoses have never been observed in fixed parenchyma cells but only in neoblasts. Perhaps this indicates that formation of new fixed parenchyma cells in normal non-regenerating animals is dependent on the activity of neoblasts.

In the mammalian brain the relationship of the neuroglia to neural elements is remarkably similar to the situation with planarian parenchyma. As enconutered with planarians, there has been much confusion as to the presence of a ground substance in central nervous tissue. Hess's (1953) claim to have demonstrated a PAS-positive ground substance in the gray matter of brain tissue was denied by subsequent investigations (Dempsey and Luse 1958, Gerschenfeld et al. 1959). The electron microscopical studies carried out by these and other authors revealed that as with the planarian parenchyma, there was not enough intercellular space present to account for the PAS-positive reaction. For this reason, the PAS-positive reaction is thought to be due to the cytoplasm of certain glia cells. It has also been suggested that the cell membranes of excessively folded neuroglia cell processes might give a PAS-positive reaction in light microscopical sections (Dempsey 1958, p. 113). In electronmicrographs, there is a close morphological similarity between fixed parenchyma cells of planarians and oligodendroglia cells or protoplasmic astrocytes. It should be pointed out that in electronmicrographs there still is some controversy as to the identification of these glial cells (Hartmann 1959, p. 85). Tissue culture methods applied to neuroglia cells have revealed a highly dynamic activity of these cells (Pomerat 1958). This activity is thought to be of great physiological significance. The glia cells undoubtedly play an important role in water and electrolyte metabolism in the brain (Gerschenfeld at al. 1959). It is thought that the cytoplasm of the glia cells may be the path of transport of materials from blood to the nerve cells and constitute part of the blood-brain barrier (Wolf 1958). It is often seen that capillaries in the brain are completely surrounded by the cytoplasm of neuroglia cells (Hartmann 1958, Pease et al. 1958, p. 58).

In planarians, where a circulatory system is lacking, the parenchyma cells are often found completely investing the cells of the excretory system, the protonephridia, and they cover the basal parts of the gastrodermal nutritive cells.

The morphological and topographical similarity between the neuroglia cells and the fixed parenchyma cells may reflect a common functional situation. In both cases they represent a continuous cellular system penetrating a solid and closely packed mass of cells practically devoid of intercellular space and ground substance. A function as a mediator of transfer of metabolites seems indicated. Other cell types with similar functions may be the Müller supportive cells in

the retina and the Sertoli cells in the testis. The electron microscopical picture of these cells resembles that of the fixed parenchyma cells.

Some of the morphologic and accordingly circumstantial evidence of the transport function will now be briefly discussed. As a circulatory system is not developed in planarians, another mechanism must be present to account for the distribution of metabolites and for the transfer of excretory products to the protonephridia. Digestion in planarians is mainly intracellular (WILLIER et al. 1925, JENNINGS 1957, ROSENBAUM and ROLON 1960). The digestion takes place in food vacuoles in the gastrodermal phagocytes. This process has recently been studied by methods of enzymatic histochemistry by ROSENBAUM and ROLON (1960). The food vacuoles contain at least three of the six or more hydrolases associated with lysosomes (see below). Besides the food vacuoles numerous pinocytotic vesicles are present in the cytoplasm of the phagocytes (PEDERSEN 1961). A reasonable picture can be given of the events in the phagocytes during digestion but virtually nothing is known concerning the subsequent fate of the metabolites or the mechanism of their transportion. In *Planaria vitta* it is certain that fat is present in large amounts in the cytoplasm of the fixed parenchyma cells but knowledge as to how it has arrived there is nil. There are at least two possibilities for the mechanism of transfer from the phagocytes to the surrounding parenchyma cells. Low molecular weight metabolites may diffuse or become actively transported without any participation of cellular membranes. Another possibility is a transfer involving "membrane flow and membrane vesiculation" as postulated by BENNETT (1956a). This mechanism has been observed in many cell types, e.g. amoeba (HOLTER 1959a, 1959b), capillaries (PALADE 1960), macrophages (ESSNER 1960) and seems to be a highly important and fundamental mechanism for transport in cells. The vesicles frequently found in the parenchyma cells might point to such a process as a means of transport from the gastrodermal cells to these cells. However, an actual proof of this cannot be given at the moment. Only few regions between parenchyma cells and phagocytes have been explored. Since most of the planarians examined had been starved for 3—6 days prior to fixation, it would be anticipated that vesiculation activity would be reduced from normal. In a future study on the histophysiology of the parenchyma this transport problem will be examined more thoroughly.

Besides the small vesicles discussed above, another group of cytoplasmic structures believed to be engaged in transport and intracellular digestion are found. These are the lysosomes. This heterogeneous group of subcellular particles have recently attracted a good deal of attention (BENNETT 1956b, NOVIKOFF 1959a, 1959b, DE DUVE 1959, ESSNER 1960). Originally "the name lysosome designates a special class of particles present in suitably prepared rat liver homogenates" (DE DUVE 1959). Fractions containing these lysosomes are rich in at least six hydrolases, among these acid phosphatase, which is generally used to follow the lysosomes. The morphological appearance of these particles is rather polymorphic as revealed by electron microscopy of isolated "pellets" or intact sectioned cells. They vary in size and are generally surrounded by a single dense membrane. Their content is dense and often heterogeneous. Lysosome-like structures have been found previously in liver ("dense bodies", "peribiliary bodies"), kidney, macrophages and other vertebrate tissue cells (DE DUVE 1959).

From the above considerations, it is suggestive that the dense membrane bounded structures found in planarian parenchyma cells belong to the group of lysosomes. There is also some evidence of a scattered but faint acid phosphatase and a non-specific esterase activity in the parenchyma, perhaps related to the presence of these structures. There is no completely decisive proof of this, however, as localisation is difficult in frozen sections of planarians. In a few cases structures more of the appearance of small food vacuoles were observed in the cytoplasm of the fixed parenchyma cells. It seems highly probable that the presence of lysosomes in these cells is related to intracellular digestion. The question of the origin and disposal of the lysosomes is possibly significant for the problem of food transport as well.

In conclusion, it can be stated that there is rather strong indirect evidence for an active participation of the fixed parenchyma cells in transport and intracellular digestive processes. Further elucidation of this problem will require a careful study of the parenchyma with histochemical and electronmicroscopical methods. Introduction of tracer methods will be useful. It will be absolutely necessary to use planarians in different nutritional states. It is possible to starve planarians for more than one year and a study of the parenchyma at selected intervals during this period of time will certainly be rewarding.

Summary

Cytological, histochemical, electronmicroscopical and *in vivo* dissociation methods have been employed in studies on the connective tissue in two planarian species. Two cell types belong to the connective tissue: the "free" neoblast and the "fixed" parenchyma cell.

The fixed parenchyma cells penetrate and bind together all structures between the epidermis and gastrodermis. The cells are very large and provided with many attenuated processes. The cytoplasm exhibits a strong PAS-reaction, presumably due to a high content of neutral polysaccharides. The cytoplasmic RNA is only present in small quantity. There are indications of very low activities of oxidative enzymes and hydrolases. Correlated light microscopical and electronmicroscopical examination revealed that most of the PAS-reactive material is intracellular.

In electronmicrographs the cytoplasm of fixed parenchymal cells is generally pale and contains few, irregularly distributed mitochondria and scanty Golgi material. In *Planaria vitta* a large number of lipid droplets are present. This is not the case in *Dugesia tigrina*. Many vesicles of several types are observed. Also the occurrence of lysosome-like structures is a characteristic finding.

The fixed parenchyma cells do not form a syncytium, and desmosomal contacts with other cells have not been observed. The extracellular space is extremely small, limited to the narrow space between two apposing cell membranes. In few places it is slightly widened. Little or no ground substance is present.

Connective tissue filaments are found in the highly developed subepidermal basement membrane. The finding of filaments in many portions of the narrow intercellular space is of considerable interest.

The great flexibility and probable great functional significance of the fixed parenchyma cells is discussed in relation to modern concepts of cellular transport mechanisms.

It is suggested that the connective tissue in planarians perhaps is one of the most primitive expressions of connective tissue in the animal kingdom.

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SOME OBSERVATIONS ON THE FINE STRUCTURE OF PLANARIAN PROTONEPHRIDIA AND GASTRODERMAL PHAGOCYTES

By

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With 9 Figures in the Text

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Introduction

Electron microscopy has recently made important contributions to the understanding of some of the ways in which substances can be transferred into cells and subsequently transported within the cytoplasm. In the last few years the significance of pinocytosis as a means of uptake of fluids, both at the lightmicroscopic and submicroscopic level, has been realized (Bennett 1956a, Holter 1959a, 1959b, Palade 1960). Closely related to the process of pinocytosis is phagocytosis (Holter 1959a, 1959b, Farquhar and Palade 1960). The fine structural aspects of this phenomenon has recently attracted a great deal of attention (Essner 1960, Karrer 1960).

During phagocytosis, and often in pinocytosis as well, an intracellular digestion of the ingested material takes place. A close relationship between this process and the presence of certain cytoplasmic particles (lysosomes) seems to be indicated (Bennett 1956b, de Duve 1959, Novikoff 1959, Rosenbaum and Rolon 1960, Essner 1960).

Up till now, relatively few cell types have been investigated with the electron microscope with respect to their pinocytotic and phagocytotic activity. Moreover, knowledge of the distribution of lysosome-like structures in animal cells is at present in an unsatisfactory state (de Duve 1959). With the exception of the amoeba, which has played an important role in the development of the concept of pinocytosis (Mast and Doyle 1934, Holter 1959a and 1959b), the invertebrates have attracted little attention for these points of view.

The main purpose of the present paper is to extend the knowledge of some aspects of the fine structural morphology of pinocytosis and phagocytosis and the distribution of lysosome-like particles to two invertebrate tissues, the gastrodermis and the excretory tubules of planarians.

Material and methods

Two species were employed, *Planaria vitta* and *Dugesia tigrina (= Planaria maculata)*. [Phylum: Platyhelminthes, class: Turbellaria, order: Tricladida after Hyman (1951)]. The individuals used had been starved for 3—6 days. Whole animals were fixed at 0° C in 2—3% osmium tetroxide buffered with s-collidine buffer (Bennett and Luft 1959) for 2—4 hours. They were quickly dehydrated in ethanol (less than one hour total) and cut into smaller pieces during dehydration. The tissue was embedded in an epoxy resin ("Epon") according

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to the method of LUFT (in press) and cut with a Porter-Blum microtome using glass knives. Sections displaying interference color of light yellow to gray were picked up on copper grids (200 mesh) covered with a thin carbon film. They were first examined in a phase contrast microscope using the high dry lens. Satisfactory sections were subsequently stained in 2% uranyl acetate for 2—4 hours. The grids were observed in RCA-2A or RCA-2C electron microscopes provided with specially designed external power supplies, objective lens apertures and objective lens compensators. All photographs were taken on "Kodak Fine Grain Positive Film" using the method of WOOD and HOWARD (1959).

Results

1. Comments and observations on the protonephridia

The term protonephridium denotes an excretory tubule opening on the body surface through a nephridiopore and ending blindly in the interior of the animal. In one type of protonephridium the inner closed end consists of a terminal cell, a flame bulb, the ciliary tuft of which creates a current of water directed distally down the tubule.

The historical development of knowledge of tricladian protonephridia, has been described by IIJIMA (1884), CHICHKOFF (1892), WILHELMI (1906) and VON GRAFF (1912—1917). The most important contributions to knowledge of the protonephridia in fresh water triclads were provided by LANG (1881), IIJIMA (1884), CHICHKOFF (1892), WILHELMI (1906), MICOLETZKY (1907) and UDE (1908). These investigators obtained their information by painstaking observations on living, young, unpigmented animals or on serial sections. Different species vary much in the ease by which their excretory system can be visualized. In some species it is extremely difficult to observe the protonephridia because of the presence of heavy pigmentation or of excessively thin-walled tubules which may collapse during histological processing. In the course of the present study it was realized that even in species where it has proved nearly impossible to observe the excretory sytem by classical histological methods, sections of specimens fixed in osmium tetroxide, embedded in methacrylate or epon and sectioned at 0.5 to 2 μ allow a precise and unambiguous visualization of the protonephridia.

The scanty knowledge of the histological construction of the protonephridia has been summarized by VON GRAFF (1912—1917), REISINGER (1928—1933), BRESSLAU (1928—1933) and HYMAN (1951). The very tortuous tubules consist of a one layered epithelium, in which no cell limits can be discerned with the light microscope. The investigators mentioned above could not determine if the lumen was intra- or intercellular. The cytoplasm was found to be moderate acidophilic, of a fine granular or homogenous appearance and provided with ovoid, deeply staining nuclei. The diameters of the tubules are described to vary widely both within same animal and between different species. The terminal cells can be isolated and examined in the living state, and are hence better known than the tubule cells. Single terminal cells provided with one ciliary tuft are called *flame cells*. Occasionally a single cell may form several ciliary tufts and the term *flame bulb* is then more appropriate (HYMAN 1951). The cilia project from the concavity of the bulbous closed end of the flame cells. Sometimes basal bodies can be discerned. A nucleus is found and the cytoplasm has a vacuolar appearance.

Phase contrast microscopical observations on isolated terminal cells obtained by trypsin dissociation of planarians corroborated the description given above.

The terminal cells were of the flame cell type with one ciliary tuft. The cells were found to be relatively small in both species.

In vivo experiments seem to indicate that most of the ciliary action in dissociated preparations is due to flame cells or epidermal cells. In a few cases, however, there was some indication of a ciliation of part of a tubule other than the terminal cell. In such cases the cilia appeared to be much longer than those in the flame cells. The cilia in the isolated flame cells are somewhat shorter than the length of the cell itself. These in vivo observations are helpful in the interpretation of the electron micrographs.

It should be mentioned that recent unpublished observations of BETCHAKU employing his impregnation method for nerve fibers (BETCHAKU 1959) has revealed a strong ciliation of some of the tubules in several species, also in *Planaria vitta*. The cilia often seemed to be extremely long. Because of the small size of the flame cells and the cilia in these cells, BETCHAKU's findings makes it unlikely that the long cilia belong to flame cells.

The present study has not contributed to knowledge of the distribution of the tubules in the species examined but has been able to provide a reliable method for this purpose (see above). At this point it seems relevant to mention that personal preliminary observations strongly suggest that at least parts of the protonephridial system exhibit an intense reaction for alkaline phosphatase. Moreover, there are indications that this reaction delineates only the protonephridia and not other cell types in the species here examined.

The fine structural findings will now be reported. For reasons previously mentioned (PEDERSEN, 1961), *Dugesia tigrina* is much better suited for electron microscopic examinations than is *Planaria vitta*. Accordingly most of the findings refer to the former species. The few observations made on *Planaria vitta*, however, corroborate the following findings.

Previous light microscopical work on fresh water triclads did not permit a decision as to whether or not there is a regional differentiation of the excretory tubules as has been described for a terrestrial triclad by DANIELLI and PANTIN (1950). In the species examined in the present study, similar cytoplasmic fine structural features are displayed in the tubules without cilia, the tubules with cilia and in the finest ramifications of the tubules (Fig. 1), but obviously there is a regional differentiation based on the presence or absence of cilia.

During the major part of the present work it was not realized that a tubular ciliation was present, and accordingly sections displaying luminal cilia were considered to represent sections of flame cells. However, this criterion does not longer hold true and it is very difficult to decide whether a given ciliated region belongs to a flame cell or a tubular cell. No description will be given of the structure of the flame cell in the present study as this subject is dealt with by WETZEL (in preparation). The cytoplasm of the flame cell seems however, to differ from the tubular cells in being much lighter, possessing many basal infolded cell membranes and many mitochondria.

Electron micrographs of cross or longitudinal sections of the tubules show that the wall is formed of a single layer of cells (Fig. 2, 3). A general feature is the presence of at least two cells in a cross section. This is found even in the smallest and most narrow tubules. Characteristically the pairs of apposed cell

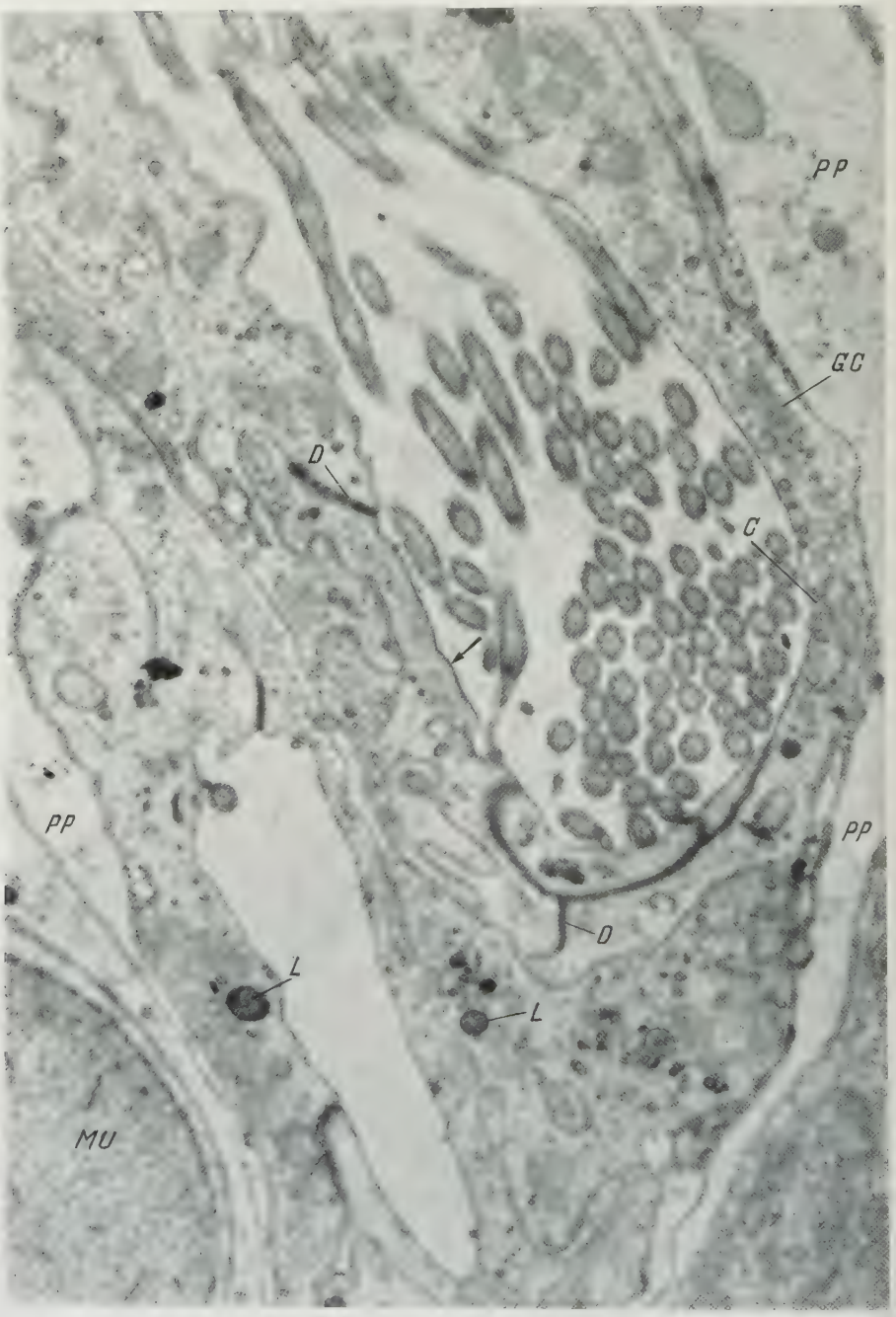

Fig. 1. Section through a ciliated portion of a protonephridium from *Dugesia tigrina*. The protonephridial cells are surrounded by pale processes of fixed parenchyma cells (*PP*). The dense cell membrane of the luminal border is seen in several places (arrow). Many small vesicles, both dense-walled and without dense membranes, are seen. Lysosome-like dense structures are present several places (*L*). Desmosomal regions are evident at *D*. Mitochondria with irregular cristae and a Golgi region (*GC*) are present. A caveola is seen (*C*). A muscle fiber (*MU*) is present in the lower left corner.
14600 ×

membranes from the two cells are lying on opposite sides of the lumen, so that approximately half of the lumen is bounded by each cell. In many instances portions of more than two cells are found in one cross section, with one cell seemingly wedged into or between others; a feature somewhat reminiscent of the organisation of the convoluted tubules in mammalian kidney (RHODIN 1958) (Fig. 1, 3). The cell membranes often have a very tortuous course, especially in the basal parts of the cells. The tubular cells are usually rather smoothly demarcated from the surrounding cells and are not provided with processes. The cell membranes bounding the basal part of the tubules are separated from apposing cell membranes by narrow intercellular spaces, a few hundred Ångström in width. This interspace may in some cases be somewhat wider (Fig. 3, arrow) but a conspicuous extracellular space is not present. This is significant in connection with the function of protonephridia. The tubules may be adjacent to cells of several different types; gland cells, muscle cells etc., but in practically all sections examined, one or more processes of the fixed parenchyma cells (PEDERSEN 1961) covers a part or the whole of the tubular surface (Fig. 1, 2).

The membrane covering the cells is about 70 Å thick and displays the unit membrane structure as described by ROBERTSON (1959) (Fig. 5). These membranes display two parallel peaks in density about 50 Å apart. The cell membrane over the luminal portion of the cell is irregular. At low magnifications it appears wider and more dense than the plasma membrane over the basal part of the cell. This may be due to the presence of an outer coating material (Fig. 2, 5). Alternate thick sections treated by the PAS-procedure display a distinct and intense positive reaction at the luminal border. The PAS-positive material might be a polysaccharide or perhaps a polysaccharide-protein complex. Small inpocketings (caveolae) are occasionally observed [Fig. 1, 3 (*C*)].

The presence of desmosomes is a general feature (Figs. 1, 2, 5). They are always found where apposing cell membranes lie close to the lumen. The cell membranes are in these places very close together (100—150 Å) and appear considerably more dense than typical cell membranes in other areas. The unit membrane structure of ROBERTSON (1959) is clearly indicated under appropriate conditions (Fig. 5). The cytoplasm bordering the desmosomal areas exhibits an increased density without obvious filamentous structure. In many instances the desmosomes display characteristics of the septate type described by WOOD (1959). As the septate structure is more clearly visualized in the gastrodermal phagocytes it will be treated in section 2 in this paper (page 621). Septate desmosomes are never observed in the space between the basal portions of the cells and the neighbouring cells.

The nuclei are slightly irregular, often ovoid, with longest diameters arranged normal to the radii of the tubules. The nuclear envelope is composed of two membranes bounding an interspace of intermediate density. Many rather coarse blocks of chromatin are found. Some of these are apposed to the nuclear envelope. In one case two nuclei are seen in longitudinal section, one on each side of the tubule (Fig. 2).

Many mitochondrial profiles about 0.4 μ in diameter and up to 1.2 μ long are present in protonephridial cells. The mitochondria conform in structure to those described by PALADE (1953). In common with other planarian mitochondria, the

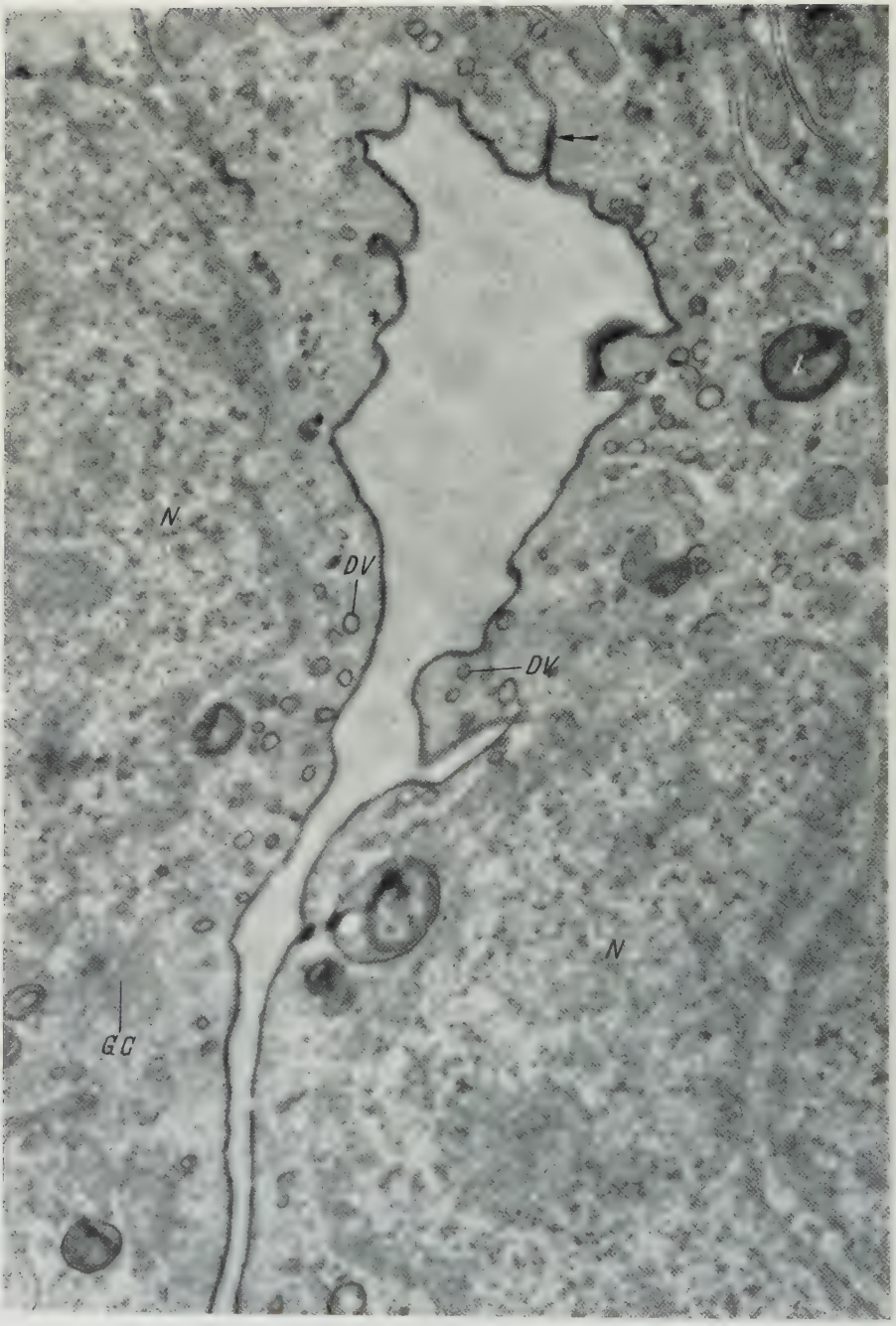

Fig. 2. Section through a protonephridium from *Dugesia tigrina*. Two nuclei (*N*) from two apposing tubular cells are seen. In the upper left corner is a process from a fixed parenchyma cell. Note the dense luminal cell membranes, the small dense walled vesicles (*DV*) and the lysosome-like structures (*L*). A conspicuous Golgi complex is present (*GC*). In the upper part of the protonephridium a desmosomal area is present (arrow). 23900 ×

cristae are lamellar and rather irregular, often running in the direction of the longitudinal axis. The mitochondrial matrix is rather dense.

Fig. 3. Section of a protonephridium from *Dugesia tigrina*. Note the many vesicles in the cytoplasm. The arrow points to an extracellular space which, incidentally, is the largest one observed in these studies of planarian tissues. A caveola (*C*) is seen. 27 300 ×

A Golgi region is present in the protonephridial cells. This organelle consists of a pile of flattened sacks, the profiles of which appear as two parallel dense

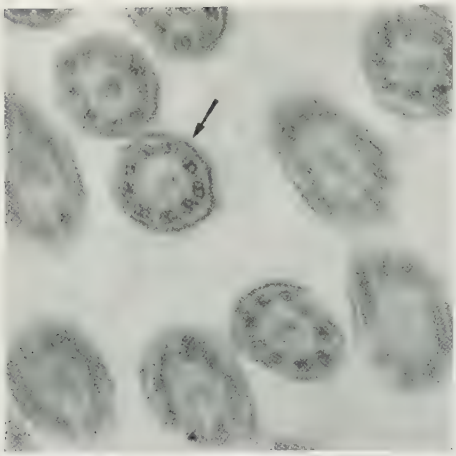

Fig. 4. This picture shows the construction of a cilium from a protonephridium. See the text. 55 000 ×

lines closed at the ends (Figs. 1, 2). Many small vesicles are found associated with these Golgi membranes.

A rough-surfaced endoplasmic reticulum was not seen. However, in the cytoplasmic matrix there is a widespread occurrence of small particles about 150 Å in diameter, probably RNA-granules (Palade granules). These granules usually are found in small groups. There was no clear evidence of an occurrence of a smoothsurfaced endoplasmic reticulum unless the vesicles just to be described might be classified as belonging to this system.

A characteristic feature of the protonephridial cytoplasm is the presence of many vesicular structures of varying size and appearance. Two types of vesicles can be discerned. One type is surrounded by a membrane, about 70 Å thick,

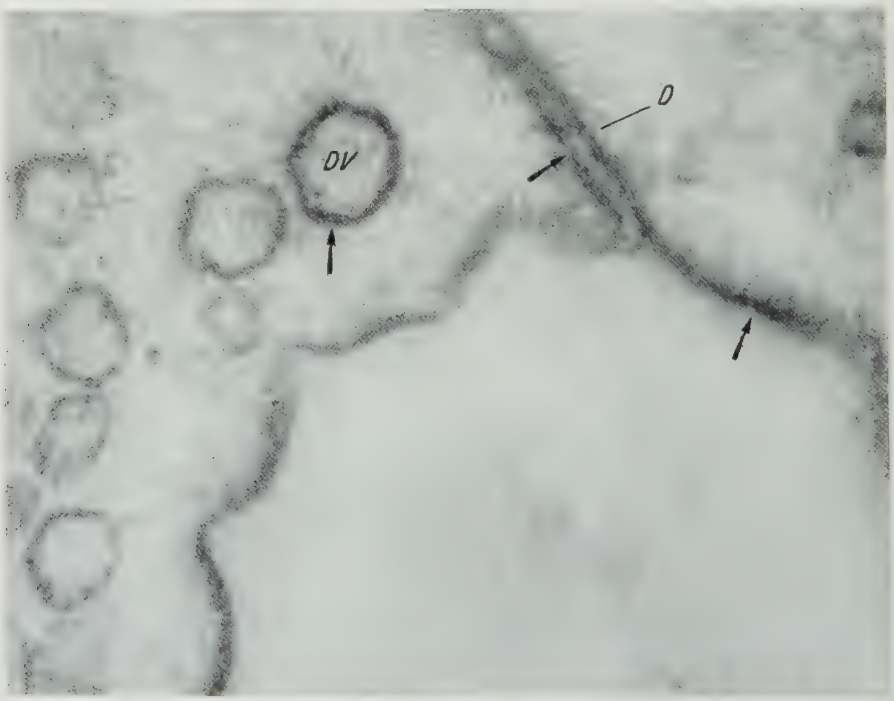

Fig. 5. Lumen and surrounding cytoplasm of a protonephridial tubular cell (*Dugesia tigrina*). Two adjacent cell membranes are seen in the top of the lumen exhibiting a desmosomal region (*D*). Dense walled vesicles are present in the cytoplasm (*DV*). The unit membrane structure is distinctly seen both in the luminal cell membrane, in the two adjacent cell membranes and in the vesicles (arrows). 126 400 ×

showing the same density as the usual cell membranes. The membrane surrounding the other type is considerably denser and resembles the cell membrane on the

luminal surface of the cell (Figs. 2, 5). There is evidence of intermediate types between these two extremes. The vesicles are about 300—800 Å in diameter. They are approximate circular in profile and the membrane is generally smooth, but is occasionally slightly wrinkled. A unit membrane structure can sometimes be discerned (Fig. 5, left arrow). The frequency with which these vesicles are found varies from section to section, as do the types of vesicles found. The contents of the vesicles are homogeneous and of moderately density. The appearance of the vesicles, provided with the dense membranes, suggests that they may have formed from the luminal cell surface by a membrane vesiculation process, as suggested by Bennett (1956a). The density of the membrane may then partly be due to the polysaccharide-protein coating referred to above. A further indication of a possible engulfing capacity of the luminal cell membrane is the apparant undulation of this structure. Further comments on these vesicles will be left for the discussion.

Dense polymorphic bodies occur in the cytoplasm rather frequently. They vary much in size, from 0.07 μ to 0.3 μ (*L*, Figs. 1, 2). The bodies are surrounded by a dense membrane of the same appearance as the dense membranes surrounding some of the cytoplasmic vesicles. They have a rather inhomogeneous content, often with portions of high density. This morphology suggests that the structures are examples of lysosomes (de Duve 1959, Farquhar and Palade 1960).

Cilia encountered in the protonephridia conform in general to the description of Fawcett and Porter (1954). Each cilium is bounded by a membrane about 70 Å thick (Figs. 1, 4) and displays a central pair of cylindrical filaments and nine pairs of peripheral filaments. The diameter of the central and peripheral cylinders is about 200 Å. Occasionally the attachment regions and basal bodies of the cilia and ciliary rootlets were observed (Fig. 1, upper left), but too few data are yet available to justify comments on these structures.

2. Observations on the gastrodermal phagocytes

The tricladian gastrodermis has received a great deal of attention and many histological details are known about this structure. Iijima (1884), Chichkoff (1892), Ude (1908) and several other investigators are responsible for the excellent classical descriptions which have been summarized by von Graff (1917 –1922). The gastrodermis has also been an object for physiological experimentation with respect to the mechanism of digestion (Metchnikoff 1878, Willier et al. 1925, Jennings 1957, Rosenbaum and Rolon 1960).

Two cell types are found in the single-layered gastrodermis: the phagocytes, which are most abundant, and the club cells or Minotian gland cells, which are present in varying numbers. In this paper only the phagocytes will be described. These cells are highly polymorphic. In animals fixed soon after feeding the cytological picture is very complicated. Phagocytes are advantageously examined after about one week's starvation. In *Dugesia tigrina* the nucleus is situated in the basal part of the cell and is provided with a prominent nucleolus. The cytoplasm shows affinity for both anionic and cationic dyes and contain many acidophilic droplets. Lipid droplets are often abundant. The free ends of the cells often bulge into the lumen. When examined with the light microscope it is sometimes difficult or impossible to decide if cell limits are present. Accordingly many

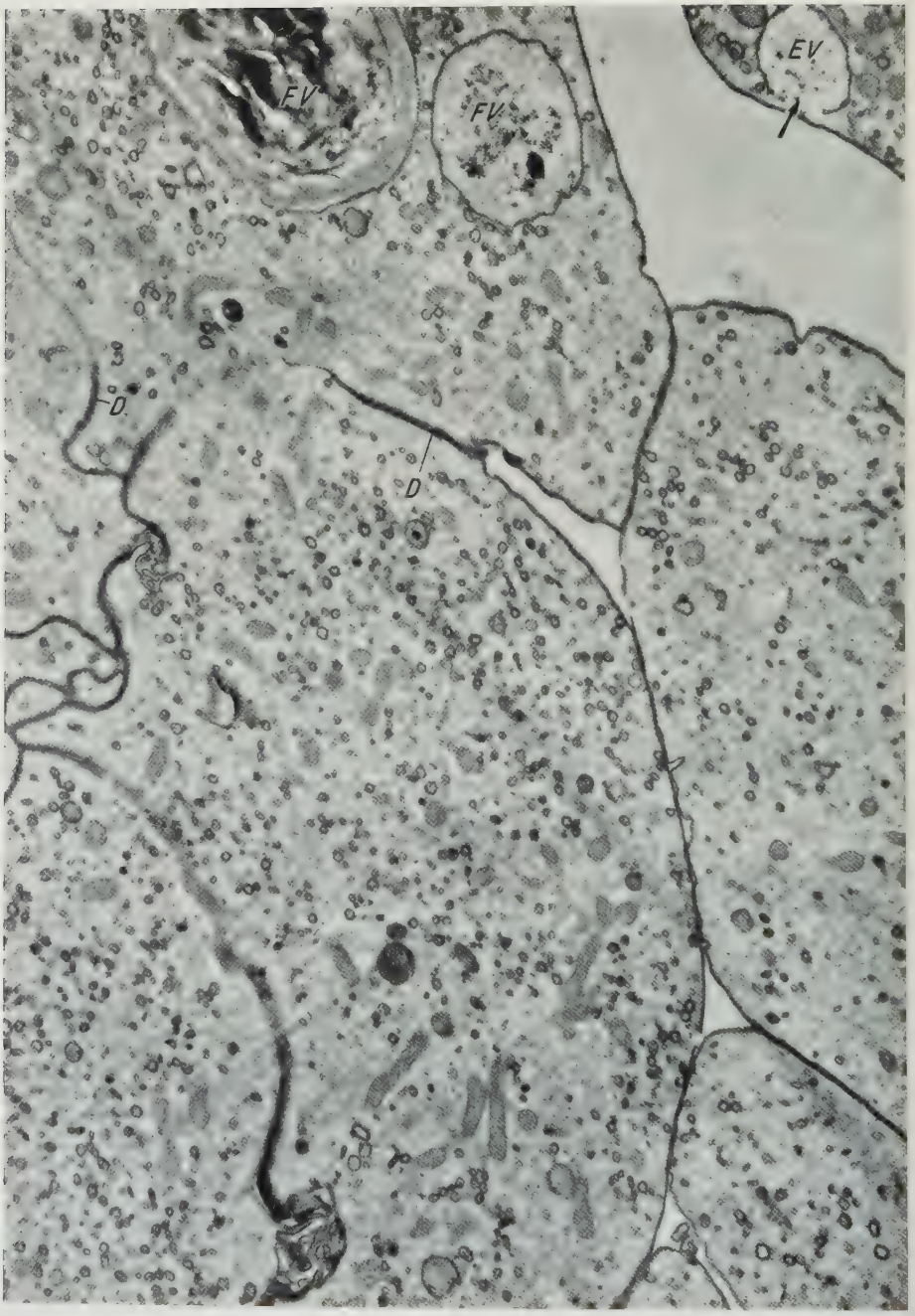

Fig. 6. Low magnification of gastrodermal phagocytes (*Dugesia tigrina*). The apical parts of several cells are seen. Note the dense luminal cell membrane complex. This is due in part to a PAS-positive coating material. In the cytoplasm are seen three large food vacuoles (*FV*). The cytoplasm contains a high number of small dense-walled vesicles. Note the desmosomal areas (*D*). The arrow points to a break in the membrane of the food vacuole. 9100 ×

investigators have described the gastrodermis as syncytial or have attributed a reversible syncytial nature to it (Reisinger 1959). The gastrodermis of *Planaria vitta* exhibits a confusing picture at all stages of feeding and starvation.

Isolated phagocytes from both species were studied, using phase contrast microscopy after trypsin dissociation. The individuality of the phagocytes was evident, and an amoeboid movement of these cells was often observed. The phagocytes usually rounded up slightly, the nucleus with the prominent nucleolus was dis-

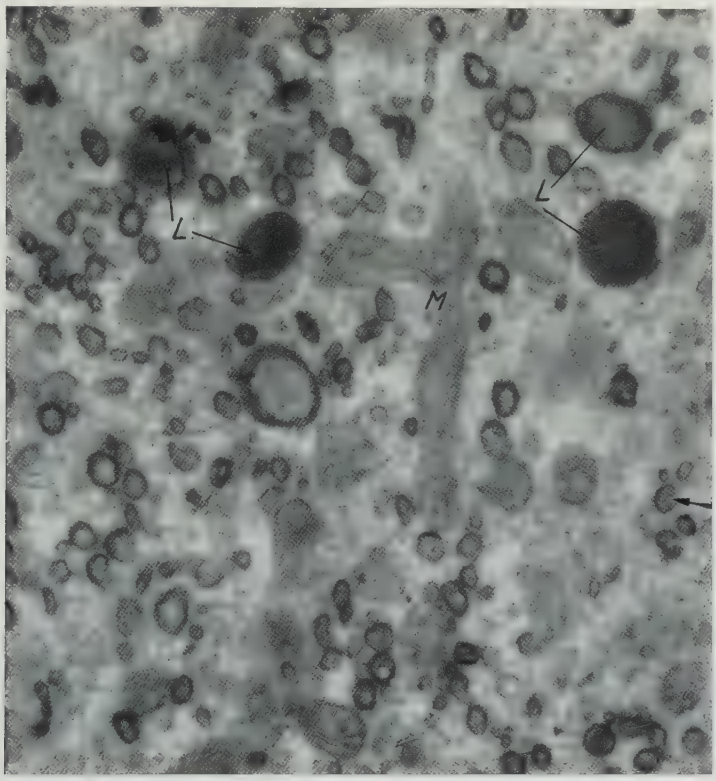

Fig. 7. Higher magnification of a part of the luminal cytoplasm of a phagocyte. Dense walled vesicles and thin walled vesicles are seen. Note the dense lysosome-like structures (*L*). The arrow points to a fragmented dense walled vesicle. Mitochondrion: *M*. 27 900 ×

placed to the center and the lipid droplets and inclusion bodies took central positions. A hyaline ectoplasm then became prominent and the formation of pseudopodia took place in this region of the cytoplasm.

The following description of the fine structural findings will mainly be restricted to the cytoplasm and luminal regions of these cells. All pictures examined represented frontal or nearly frontal sections.

The cell membrane displays the usual thickness of about 70 Å and frequently shows a very tortuous course. The cells often seem to be wedged into each other in intricate arrangements. Hence it is not surprising that these cells in the past have been described as being syncytial. The region of the cell membrane facing the lumen seems to be thicker and is considerably more dense than the other regions of the cell membrane (Figs. 6, 8). A similar explanation as given for the

luminal part of the protonephridial cell membrane also may apply here: namely the presence of a coating of a polysaccharide or polysaccharide-protein complex. A

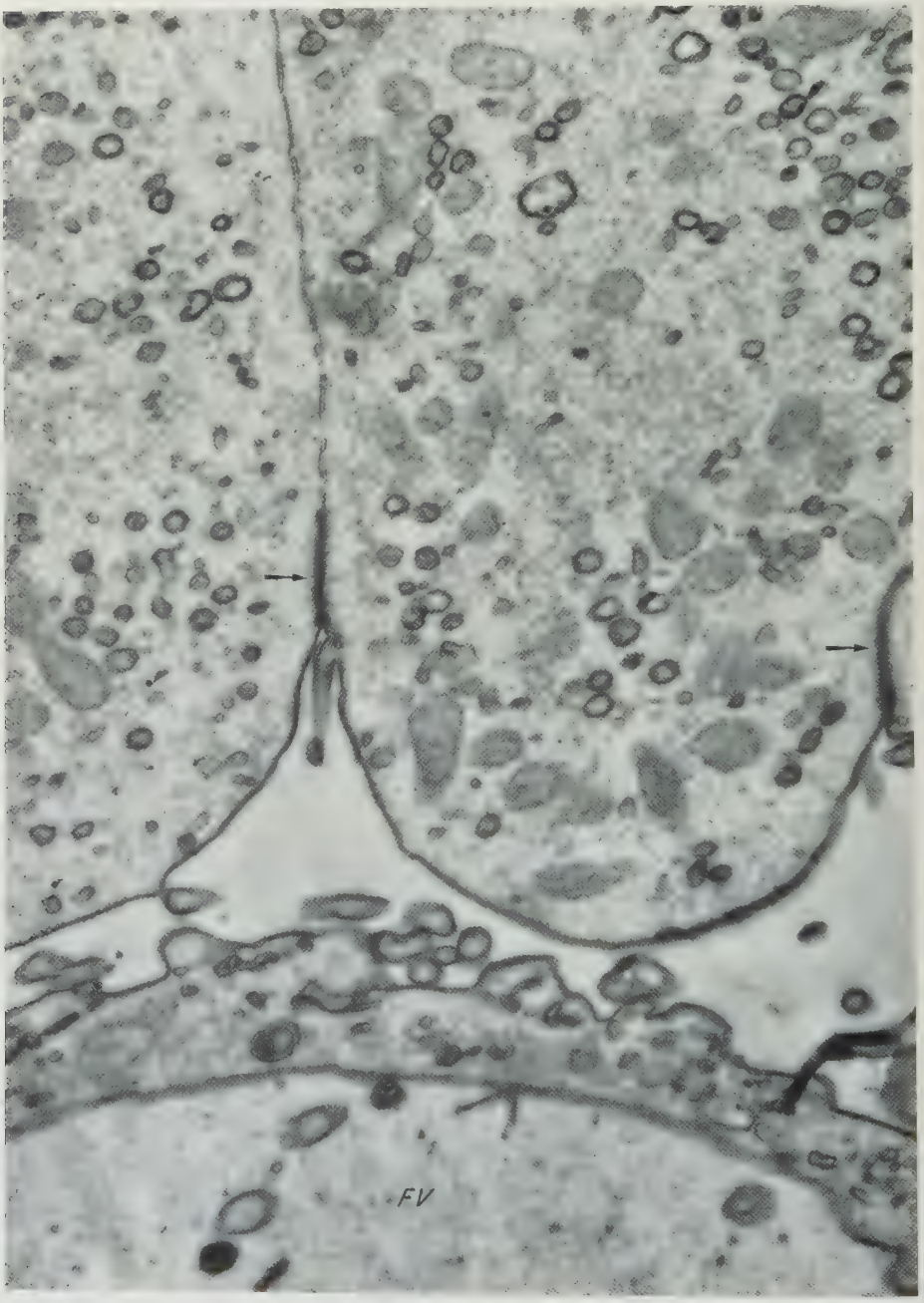

Fig. 8. In the upper part are the apical regions of two gastrodermal phagocytes (*Dugesia tigrina*). In the bottom a section through a tip of a phagocyte with a huge food vacuole (*FV*). Notice the finger like projections towards the lumen. In the cytoplasm are many small vesicles, partly dense walled and partly thin walled. Septate desmosomes are present at the luminal border (arrows). 22 500 ×

strong PAS-reactivity of the luminal border is found in adjacent thick sections. In most places the luminal cell membrane is straight but occasionally small finger-like or more complex projections are seen (Fig. 8). Also inpocketings (caveolae) are observed but more rarely.

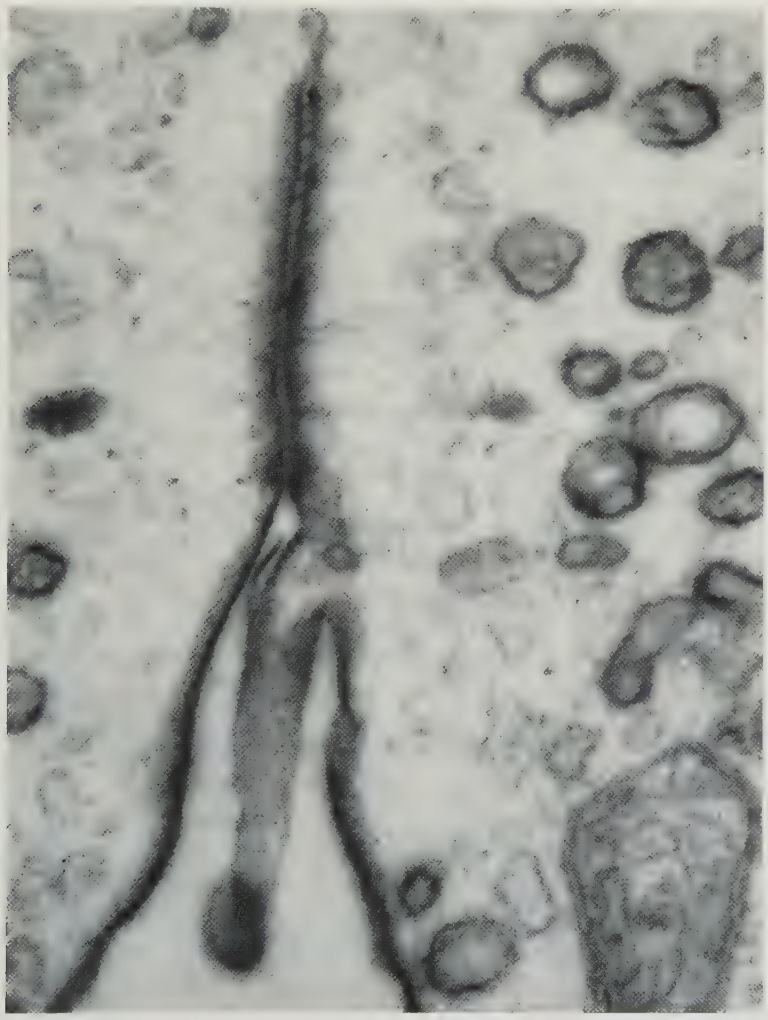

Fig. 9. A magnified portion of Fig. 8 showing the structure of a septate desmosome. 78000 ×

Desmosomal regions are generally found (Figs. 6, 8). They are always observed where cell membranes approach each other at the lumen and occasionally in other locations. The cell membranes and cytoplasm in the desmosomal regions show an increased density. The desmosomes often seem to be of the septate type, as is the case of the tubular cells in the protonephridia (Fig. 8, arrow). Wood (1959) also found septate desmosomes to be present in the gastrodermis.

In these cases dark lines are seen traversing the interspace, running at right angles to the two cell membranes (Fig. 9). No attempt was made to elucidate

further the detailed morphology of these interesting structures, as it was apparent that the preparation method employed was not ideally suited for this purpose. According to WOOD, staining with phosphotungstic acid during dehydration greatly facilitates the study of these attachment lamellae.

Numerous mitochondria are present in the cytoplasm. They have diameters of about 0.2 μ. The cristae are irregularly arranged, but seem predominantly to be parallel to the longitudinal axis.

A Golgi-complex is observed, displaying features similar to those of the protonephridial cells.

Very few free RNA-particles (Palade granules) were observed in the luminal part of the phagocytes. A few profiles of a smooth surfaced endoplasmic reticulum were seen. In the basal region a rough surfaced endoplasmic reticulum is present and relatively highly developed.

A characteristic feature of the phagocytes is the presence of an enormous number of vesicles. Two types can be distinguished, as in the case of the protonephridial cells (Figs. 6, 7, 8). Predominant and most conspicuous are vesicles 500—1500 Å in diameter surrounded by a dense membrane. Many vesicles possessing a considerably less dense limiting membrane are also present. These often have a smaller diameter. Occasionally the limiting membrane of the dense-walled vesicles display discontinuities (Fig. 7, arrow). This could, of course, be an artefact, but the phenomenon might be of significance as a mechanism for emptying the contents of the vesicles. The contents of the vesicles is generally of low density but is sometimes of a medium density.

In many cases food vacuoles were observed (Fig. 6). These structures are surrounded by a very dense membrane of the same appearance as the luminal cell membrane and that of the dense walled vesicles. The membrane is also strongly PAS-positive. The food vacuoles may be very large (Fig. 8, *FV*), but there is a pronounced variation in their size and content. It might be of interest to point out thet these structures display a high acid phosphatase activity, a finding first published by ROSENBAUM and ROLON (1960).

Apart from the food vacuoles, smaller bodies were observed, intermediate in size between the dense walled vesicles and the food vacuoles (Fig. 7, *L*). These structures also possess a dense membrane and usually have a dense content. These bodies might correspond to lysosomes (see the discussion).

A further evaluation of the interrelationships between the different vesicular structures observed here and their possible functional significance will be considered in the discussion.

Discussion

The present communication has been a presentation of some fine structural findings in two cellular systems where it is supposed that transport processes and intracellular digestive processes may play a dominant role in the cellular activities. It is now relevant to try to give some of the morphological observations a functional meaning. However, it must be stressed that no experiments yet have been done on these systems using electron microscopy as a recording device. Accordingly the discussion will have to be cautious and restricted until such investigations have been carried out.

The organ systems of planarians are held together in a very interesting way. It has been demonstrated that planarians possess a peculiar connective tissue, the main component of which consists of large, flexible, polymorphic cells containing a high concentration of neutral polysaccharides. These cells, the "fixed" parenchyma cells, are provided with many intricately arranged processes which bind together all structures between the epidermis and gastrodermis. An extracellular space is not present except for very narrow compartments about 150 Å thick between two apposing cell membranes. This space may contain extremely small amounts of ground substance and filamentous structures (PEDERSEN, 1961). No circulatory systems is present in these animals. It has been suggested (PEDERSEN, 1961) that the fixed parenchyma cells may play an important and fundamental role in the transportation and distribution of metabolites and waste products. The presence of many vesicles and lysosome-like structures might be an indication of this function.

This tissue organisation has significant bearings on the interpretation of the function of the protonephridia and the gastrodermal phagocytes.

It is a generally accepted view that the protonephridia are important for the maintenance of proper osmotic balance (PANTIN 1947, MARTIN 1958). Water excretion is perhaps the most important activity of the protonephridia. This view is supported by the fact that the protonephridial system is most highly developed in fresh-water turbellarians and is often absent in marine animals (BUDDENBROCK 1956). KROMHOUT (1943) examined a species of a rhabdocoel turbellarian, which occur in fresh water, brackish water and salt water. The fresh-water form has an extensive protonephridial system. This system is poorly developed in the brackish water specimens and is absent in the marine specimens of the same species. These observations suggest that water elimination may be an important function of the protonephridia.

The way in which excretion of water and dissolved material takes place is not known and the interpretation of the possible role of the ciliary flame in this process is also a subject of controversy (CARTER 1951, MARTIN 1958).

It has been suggested that a filtration might take place in the terminal cell (the flame cell). According to some investigators, the action of the ciliary tuft might provide a filtration pressure, though perhaps a very small one (JORDAN 1929, PANTIN 1947, MARTIN 1958). KÜMMEL (1959) made an electron microscopical study of the protonephridia in a platyhelminth, *Fasciola hepatica*. He found an interesting architecture in certain regions of the cytoplasm of the terminal cell, which in this species seems to constitute the whole protonephridium. The cytoplasm was arranged in longitudinal ridges connected with structures about 100 Å thick. He suggests that this region might represent a filtration surface. The present investigation has not aimed at correlating the morphology of the flame cell with function, as WETZEL is examining this problem (WETZEL, in preparation).

The excretory system is not surrounded by spaces containing fluid, as most persons have generally believed heretofore, but by closely apposed cells, most frequently the fixed parenchyma cells. The cytoplasm of these cells contain a high concentration of neutral polysaccharides. This cytoplasm might have a function similar to that of the connective tissue ground substance, binding and exchanging water and ions. Perhaps these cells may function as do the macroglia cells in

mammals, serving as mediators for transfer of nutrients, water, electrolytes and excretory products between cells. In the brain the glia cells might be mediators for transfer between nerve cells and capillaries (WOLF 1958, GERSCHENFELD et al. 1959). In planarians the fixed parenchyma cells might be mediators between the gastrodermis, the tissue cells and the excretory system. How transfer might take place is unknown. The presence of many small vesicles in the fixed parenchyma cells suggest the possibility of membrane vesiculation and vesicular transport as a mechanism of transfer.

It is not known whether reabsorption takes place in the tubules. In the case of a terrestrial triclad, DANIELLI and PANTIN (1950) found a complicated excretory system consisting of terminal flame cells connected with unciliated ducts and ciliated convoluted canals which probably connected with unciliated distal tubules. They observed a strong alkaline phosphatase reaction confined to the convoluted ciliated tubules and suggested that this might be correlated with a modification of the composition of the fluid passing down the tubules, analogous perhaps to the absorptive function attributed to the strong alkaline phosphatase activities of brush border in the small intestine and proximal convoluted tubules of the kidney in mammals. As mentioned above, preliminary studies have suggested that at least parts of the protonephridia in *Dugesia tigrina* also possess a high activity of alkaline phosphatase. A similar explanation as given by DANIELLI and PANTIN (1950) might be pertinent.

Tubular reabsorption is suggested further by virtue of the presence of the many small vesicles in the protonephridia. The similarity between the membrane of the dense walled vesicles and the luminal cell membrane suggests that these vesicles are derived from the luminal surface by membrane vesiculation (BENNETT 1956a). The coating of PAS-positive material might be important for binding of material before its uptake by vesiculation (BENNETT 1956a, HOLTER 1959a, 1959b, MARSHALL et al. 1959, BURGOS 1960). Recently ESSNER (1960) described an increased density of the phagocyte cell membrane in the immediate vicinity of erythrocyte fragments being engulfed by the phagocyte. BURGOS (1960) describes the presence of an amorphous coating rich in mucopolysaccharides on the cell membrane (brush border) of active absorbing cells such as the non-ciliated columnar cells of the ductuli efferentes testis and head of the epididymis and the cells of the proximal convoluted tubules of the kidney. He finds that injected dense colloidal particles are embedded in the coating material and subsequently brought inside the cell through membrane vesiculation. The gastrodermal phagocytes also seem to be very actively engaged in pinocytosis. Moreover, the same morphological features are encountered as in the case of the protonephridia.

Besides the pinocytotic function, the phagocytes can also incorporate solid material through phagocytosis. In planarians it is generally accepted that digestion is primarily an intracellular process. Personal unpublished observations have demonstrated a high activity of non-specific esterase in the large granules of the Minotian gland cells. Thus the secretion of these cells might be responsible for at least some extracellular digestion. However, the main part of the digestion is undoubtedly intracellular (KITCHING 1956). ROSENBAUM and ROLON (1960) have studied some enzymatic aspects of this process and report on a rise in acid phosphatase activity which begins 5 minutes after feeding and

is correlated with formation of food vacuoles. It is absent in animals which have been without food for one week. Thus it is evident that the phagocytes have capacity for formation of acid phosphatase. Aminopeptidase and β-glucuronidase were also shown to be present in the gastrodermis (Rosenbaum and Rolon 1960).

The presence of acid phosphatase activity was also demonstrated in the present study and the morphology of the food vacuoles was observed. Engulfment and membrane vesiculation was indicated by the morphological (and partly histochemical: the PAS-positive reaction) identity between the cell membrane covering the luminal portion of the cells and the membrane of the food vacuoles. Similar dense coated membranes are also found in the gastrodermal phagocytes of Hydra (Wood, unpublished). The morphological features and the enzymatic histochemical reactions remind one of lysosomes (de Duve 1959) or phagosomes (Essner 1960). Smaller particles of similar morphology are found both in the protonephridia and phagocytes and these may also belong to the group of lysosomes.

Farquhar and Palade (1960) and Essner (1960) have recently discussed the nomenclature for pinocytotic and phagocytotic bodies and the problem of the idendity between phagosomes, lysosomes, "dense bodies" etc. It seems that the basic mechanisms involved in phagocytosis and pinocytosis are the same and that there is no sharp limit between the two phenomena (Holter 1959a).

The basic mechanisms may be an initial binding to the cell surface, often mediated by a coating layer of polysaccharide or a polysaccharide-protein complex, and a subsequent engulfment through membrane vesiculation. This may take place either through pseudopodia formation or through an invagination of the cell membrane (Bennett 1956a). Subsequently, hydrolytic enzymes are either formed in the vesicles or secreted into these structures (Rose 1957, Essner 1960). A hydrolytic break-down of ingested material may take place in the vacuoles or vesicles (Bennett 1956b).

The present paper has not added anything new to these concepts but has extended the knowledge of the presence of these mechanisms to two interesting cellular systems. Experimental work with tracers and recording with the electron microscope may elucidate further aspects of the pinocytotic and phagocytotic mechanisms in these cell types.

Two other interesting phenomena deserve brief comment. Though it has been claimed that the protonephridia and the gastrodermal cells might form syncytia, this was found not to be true. A more detailed discussion of this subject has been given in another paper (Pedersen, 1961).

The ubiquitous presence of septate desmosomes at the luminal border of the protonephridia and of the gastrodermal cells indicates an important functional role for these structures. Wood (1959) has suggested that septate desmosomes may function as a permeability barrier. In Hydra they seem to defend the intercellular environment. A similar explanation seems indicated in the present case.

Summary

The paper contains a description of some of the fine structural features of protonephridial tubular cells and gastrodermal phagocytes in a flatworm, *Dugesia*

tigrina (= Planaria maculata). Special emphasis has been given to the morphological features suggesting pinocytotic and phagocytotic activities.

In vivo dissociation methods and electron microscopy were employed. An epoxy resin "Epon" was used for embedding.

Except for the occurrence of cilia in some parts of the tubules the fine structure of different regions in the tubules seem to be rather similar. High alkaline phosphatase activity is present in at least some regions of the protonephridia. No circulatory system is present, and the protonephridia are surrounded by the narrow interspace between two apposing cell membranes. A conspicuous extracellular space is not found in the animals.

The cell membrane in both the protonephridial and phagocytic cells appear very dense over the luminal portion of the cell, due to a coating of a PAS-positive material. Vesicles surrounded by a similar membrane are found in the cytoplasm of both cellular systems, and they are interpreted as originating from the cell membrane by membrane vesiculation. Lysosome-like structures are present. The morphology of the food vacuoles in the phagocytes is rather similar to the lysosomes. Both kinds of bodies are surrounded by a dense, coated cell membrane. Septate desmosomes are frequently found in both cellular systems adjacent to the luminal border of the cells.

A functional interpretation of some of the morphological findings is tentatively given.

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Aus dem Zoologischen Institut Gießen

POLARISATIONSOPTISCHER NACHWEIS KRISTALLINER FETTARTIGER STOFFE IM HAARMARK

Von

W. J. Schmidt

Mit 5 Textabbildungen

(Eingegangen am 17. November 1960)

Es ist allgemein bekannt, daß die Haare des Menschen und der Säuger in natürlichem Zustande fetthaltig sind, was sich in verschiedener Art nachweisen läßt (s. bei Biedermann 1930). Jedoch wurden die Fettstoffe meines Wissens bisher im Haar und insbesondere in seinen Markzellen niemals morphologisch nachgewiesen. Daher möge im folgenden über einschlägige Vorkommnisse bei Mensch und Säuger berichtet werden.

1. Vorkommen von Kristallen fettartiger Stoffe im Haarmark des Menschen

Bei Untersuchung einer Probe dicker braunschwarzer Frauen-Kopfhaare zwischen gekreuzten Polars fielen mir im Haarmark doppelbrechende Körnchen auf, teils vereinzelt, vielfach aber den Markstrang in seiner vollen Dicke erfüllend (Abb. 1). Die Einschlüsse fanden sich nicht in allen Haaren dieser Probe und, wo sie vorkamen, nicht im ganzen Markstrang; vielmehr wechselten einschlußhaltige und -freie Strecken miteinander ab. Die geschilderte Masse liegt *in* den Markzellen: bei reichlicher Anwesenheit ist interzellulare Lokalisation an sich schon kaum vorstellbar, und aufmerksame Beobachtung läßt manchmal an gruppenweiser Zusammenordnung von Körnchen die Abgrenzung einzelner Markzellen erkennen.

Zur näheren Prüfung der genannten Einlagerungen ist das Haar selbst auf Auslöschung zwischen gekreuzten Polars zu bringen; wenn alsdann das Keratin fast völlig dunkel wird, leuchten die Einschlüsse (Abb. 1) auf und bei leichtem Hin- und Herbewegen des Objekttisches läßt sich erkennen, wie einzelne Körnchen sich erhellen, während andere verlöschen, so daß sie also eine bestimmte Orientierung nicht besitzen.

Die *Gestalt* der Körnchen (Abb. 1a—d) wechselt; die größeren erscheinen schollig, unregelmäßig kantig begrenzt, manche kleinere aber gestreckt, an beiden Enden zugeschärft, während wieder andere abgerundeten, kugeligen oder länglichen Umriß darbieten. Unter Immersionssystemen sieht man noch winzige Teilchen solcher Art meist von nadeliger Form, oft zahlreich neben vereinzelten größeren.

Die *Polarisationsfarbe* der Körnchen ist weiß bis gelblich — sofern das Haar sich in Auslöschung befindet. In den Diagonallagen freilich werden die Körnchen zum Teil blau und orange; dies wird durch das umschließende positiv zur Haarlänge wirkende Keratin der Haarrinde verursacht, zu dem einzelne Körnchen sich in Additions- oder Subtraktionslage befinden.

Angesichts der mannigfachen Gestalt der Körnchen ist es nicht allgemein möglich, ihr *optisches Vorzeichen* anzugeben; aber bei allen von ausgeprägt anisometrischer Form fand ich es *positiv* zu der bevorzugt ausgebildeten Richtung. Dies ist hervorzuheben, weil Neutralfette gewöhnlich als nadelige Blättchen auftreten, die *negativ* doppelbrechend zur Länge (Kante) wirken.

Trotzdem spricht das Folgende dafür, daß die doppelbrechenden Einschlüsse fettartige Stoffe sind. Zunächst kann man sie in Haaren, die in Canadabalsam eingebettet sind, durch leichtes Erwärmen des Präparates über der Bunsenflamme zum Schmelzen bringen. Beobachtet man sogleich nach diesem Eingriff im

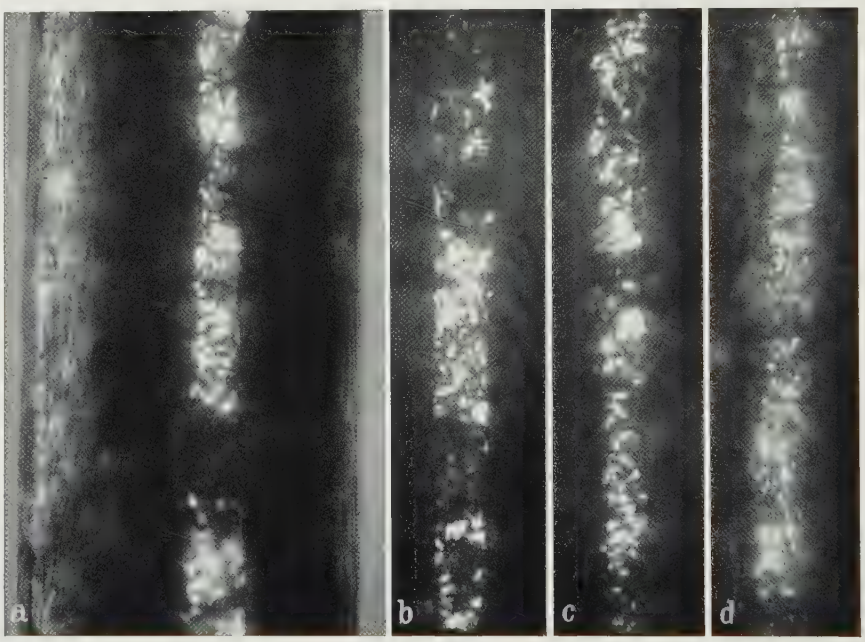

Abb. 1a—d. *Homo*, Kopfhaare, Pol. +, 380:1: fettartige Einlagerungen im Markstrang. a Abschnitt eines Haares, drehbare $\lambda/16$-Glimmerplatte; b—d Ausschnitte des Markstranges

Polarisationsmikroskop, dann sind die fettartigen Massen nicht auffindbar; alsbald aber treten die Kristalle schlagartig hervor, anfangs klein, dann rasch anwachsend. Jetzt sind nadelartige positiv zur Länge doppelbrechende Gebilde häufig. Niemals zeigten sich bei dem Übergang aus dem isotropen zum doppelbrechenden Zustand Tropfen mit Polarisationskreuz, wie sie für Cholesterinester bezeichnend sind.

Weiter sind die Einschlüsse in *Lipoidsolventien löslich*; in Haaren, die in einem Gemenge von absolutem Alkohol und Xylol zu gleichen Teilen mehrere Stunden im Wärmeschrank bei 60° C verweilten, verschwanden sie und kamen mit dem Erkalten nicht wieder zum Vorschein.

Nach diesen Feststellungen besteht kein Zweifel, daß die Einschlüsse Kristalle fettartiger Stoffe sind. Ihre nähere Natur freilich blieb ungewiß; Cholesterinester dürften bei dem Fehlen doppelbrechender Tropfen (s. oben) nicht in größerer Menge vorliegen. Auch reines Cholesterin ist nicht vorhanden, da 10stündiger

Aufenthalt in Pyridin — einem sehr guten Lösungsmittel für diesen Stoff — die Körnchen bei Zimmertemperatur nicht zum Verschwinden brachte. Nach Biedermann (1930) enthält das Hautfett der Säuger (aus Talgdrüsen, Epidermis und auch Haaren stammend), wie man vor allem aus der chemischen Untersuchung des Wollfettes bei Schafen weiß, Cholesterin und Fettsäureester verschiedener Art; beim Menschen wurden neben Fetten und Fettsäuren nur geringe Mengen von Cholesterin gefunden.

Von der gleichen Person wurden später inzwischen *ergreiste* Haare untersucht; in keinem Haar der entnommenen Probe waren die doppelbrechenden Einschlüsse im Mark nachweisbar, so daß sie also bei dem gleichen Individuum nicht zu allen Zeiten vorkommen.

Weiter wurden noch Kopfhaar-Proben von 3 weiteren Menschen untersucht. Bei einer weiblichen etwa 30 Jahre alten Person mit dicken tiefschwarzen Haaren fanden sich die doppelbrechenden Einlagerungen im Mark vielfach, wenn auch niemals so massenhaft wie in dem erst geschilderten Falle. In den Haaren einer Blondine (etwa 20 Jahre) waren die scholligen Einschlüsse sehr spärlich; jedoch stieß ich auf einige kugelige Einlagerungen mit negativem Polarisationskreuz. Im eigenen ergreisten Kopfhaar (76 Jahre) und auch in Wimper-, Brauen-, Bart- und Schamhaaren zeigten sich die Einlagerungen *nicht*. Diese Befunde stimmen im ganzen mit der Feststellung von Lenbuscher (s. bei Biedermann 1930) überein, daß brünette Individuen mehr Hauttalg abzusondern scheinen wie blonde, und daß seine Menge mit der Pubertät bedeutend ansteigt, um im Greisenalter wieder abzufallen (Lenbuscher, Linser, s. bei Biedermann 1930).

Schließlich erhebt sich die Frage, ob die Fettstoffe der Markzellen bodenständig sind oder aber von außen in das Haar eindringen und erst im Mark auskristallisieren. Ich möchte der ersten Auffassung zuneigen, also annehmen, daß die Lipoide in den lebenden Markzellen auf dem Gipfel der Haarzwiebel auftreten und damit die spätere Aufnahme von Luft in diese Zellen verhindern. Auch der Umstand, daß innerhalb reichlich Fettstoffe führender Markabschnitte einzelne Zellen nur wenig Einlagerungen darbieten oder gar frei davon sind, dürfte für deren autochthones Auftreten sprechen. Diese Fettablagerung erfolgt im Wachstum des Haares mit Unterbrechungen, wie der Wechsel von lufthaltigen und fettführenden Abschnitten im Mark des gleichen Haares bezeugt.

Es sei noch bemerkt, daß gelegentlich auch in der *Epidermicula* der Haare der erst untersuchten Probe Einlagerungen vorkamen, die mir lipoider Natur zu sein scheinen. Unter Wirkung der $\lambda/16$-Glimmerplatte (Kompensator nach Brace-Köhler) hebt sich die — im dunklen Sehfeld des Polarisationsmikroskopes unsichtbare — Epidermicula scharf von der Haarrinde ab (Abb. 2a); sie wirkt schwach positiv doppelbrechend zur Länge des Haares (W. J. Schmidt 1932)[1]. Bei geeigneter Einstellung der Glimmerplatte (Abb. 2a) tritt am Rand des auf Auslöschung gebrachten Haares der Aufbau der Epidermicula aus den platten schindelartig sich überdeckenden Zellen hervor, die im Profil strichartig erscheinen und deren an die Oberfläche reichenden Enden den bekannten gesägten Kontur hervorrufen. Die einzelnen Zellen sind durch deutliche Grenzlinien geschieden, die ich auf feinste luftgefüllte Lagen zwischen ihnen beziehen möchte.

[1] Sie löscht ein wenig schief zum Haarrande aus, nämlich parallel zur Kante der schrägstehenden Epidermiculaschüppchen.

Es finden sich nun am Haarrande (rechts, Abb. 2a, b) Stellen der Epidermicula, deren Bau leicht gestört erscheint, indem der Außenkontur etwas unregelmäßig verläuft und die Schichtdicke vergrößert ist. Diese Stellen wirken *negativ* zur Kante der Schüppchen, so daß sie sich erhellt hervorheben, wenn im übrigen die Epidermicula durch Wirkung des Kompensators verdunkelt erscheint. Auch mit der Gipsplatte Rot I erweist sich leicht das negative Vorzeichen. Wenn es gelegentlich so scheinen mag, als ob das negative Material außen der Epidermicula aufliege, so finden sich doch andere Stellen, an denen die Epidermicula in ganzer Dicke oder nur an ihrer Grenze gegen die Haarrinde die negative Doppelbrechung darbietet. Ja, unter starken Objektiven (Abb. 2c) erkennt man mit Sicherheit,

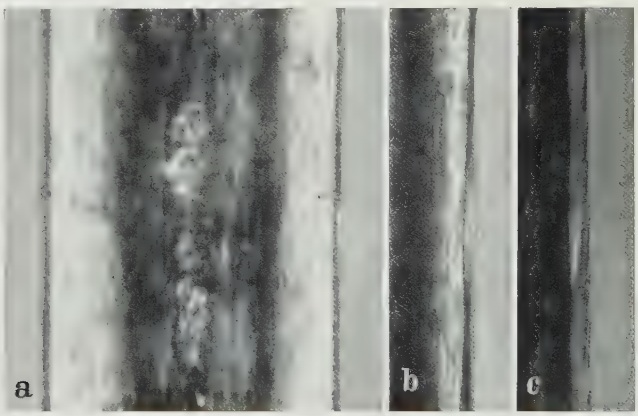

Abb. 2a—c. *Homo*, Kopfhaare, Einschlüsse in der Epidermicula, von gegensätzlichem optischem Vorzeichen wie diese; Pol. ×, drehbare λ/16-Glimmerplatte. a und b 380:1; c 830:1

daß die negative Masse *zwischen* den flachen Zellschuppen, also *interzellular* liegt. Dies wird auch deutlich, wenn das Haar sich in Additionslage zum Kompensator befindet, also die Epidermicula sich erhellt, die Einlagerung darin aber tief dunkel erscheint. An ihren Enden nehmen die eingeschalteten Schichten oft allmählich an Dicke ab. Auf Auslöschung gestellt, verschwinden sie nicht ganz, sondern zerfallen in eine Anzahl strich- oder punktartiger Teile.

Daß man die genannten Einlagerungen der Epidermicula nur am Rande des Haares, nicht auf seiner Fläche wahrnimmt, besagt nicht, sie seien strichartig. Vielmehr ergeben sich hier und dort Andeutungen, daß sie lamellär ausgebildet sind, wie es ja bei ihrer Entwicklung in den flachen Räumen zwischen den Zellschüppchen zu erwarten ist. Daher bezeugt das neutrale Verhalten ihrer Flächenansicht die senkrechte Orientierung der optischen Achse zur Lamellenebene. Die Einlagerungen der Epidermicula besitzen demnach eine positive optische Achse senkrecht zur Fläche.

Damit ergibt sich ein Hinweis auf die Fettnatur der in der Epidermicula interzellular eingelagerten Masse; denn positive Lipoidmolekeln orientieren sich senkrecht zu Grenzflächen (s. z. B. Fetteinlagerungen in der Epidermis von Schlangen, W. J. Schmidt 1959). Bei der Seltenheit und Kleinheit dieser Bildungen gelang es nicht, einwandfreie Extraktions- und Schmelzversuche zu machen, besonders auch deshalb, weil sie in manchen Präparaten alsbald nach Einschluß in Xylolbalsam zu verschwinden scheinen, wohl gelöst werden.

2. Kristalline Einlagerungen im Haarmark von Sciuriden

Zuerst untersuchte ich Grannenhaare der afrikanischen Baumhörnchen *Funisciurus lemniscatus isabellae* und *Heliosciurus poensis poensis*, an Material aus dem Museum Alexander Koenig, Bonn — wofür hier bestens gedankt sei.

Zum allgemeinen Bau der Grannenhaare der Sciuridae sei folgendes vorausgeschickt: Sie beginnen an der Wurzel dünn, schwellen dann an und laufen distal in eine feine Spitze aus. Ihre Epidermicula (Abb. 3b) besteht aus Zellschüppchen, die sich nur wenig decken, wie am Haarrande erkennbar. Die Rindenschicht ist verhältnismäßig schmächtig, kräftig ausgebildet aber der Markstrang, der, proximal und distal einreihig, in der erwähnten Anschwellung jedoch bis zu 4 Zellreihen breit wird. Wo mehrere Reihen von Markzellen nebeneinanderliegen, flacht sich das Haar gemäß der zunehmenden Breite ab. Dem Spitzenabschnitt fehlen Markzellen.

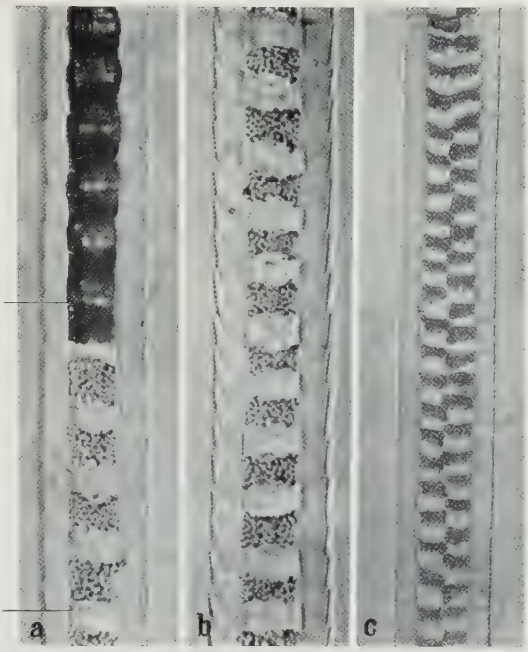

Abb. 3a—c. *Heliosciurus poensis*, Grannenhaarabschnitte mit gelbem Pigment in den Markzellen. a und b 830:1; c 380:1. a oben Markzellen mit Luft, unten mit Canadabalsam erfüllt, Pigmenthäufchen in den einzelnen Zellen und mitten im Raum dazwischen die Zellgrenzen (*G*); b Epidermicula am Haarrande sichtbar; c Haar mit zweireihigem Markstrang

In den Haaren kommen zweierlei körnige *Pigmente* vor, *Melanin* und ein *grünlichgelber* Farbstoff. Das erste findet sich in Haarrinde *und* -mark als braune *längliche* Granula, die in der Rinde entsprechend deren spindeligen Zellen nach der Haarachse ausgerichtet sind, während sie in den Markzellen, wo sie etwas gedrungener erscheinen, ungeordnet liegen. Das gelbe Pigment aber tritt nur in Markzellen auf; es nimmt, wenn vorhanden, einen mittleren Abschnitt des Haares ein. Beim Übergang von Melaninpigmentierung zu Gelbfärbung verschwinden die dunklen Granula in Rinde und Mark allmählich völlig, während zugleich in den Markzellen die gelben auftreten und immer zahlreicher werden, so daß sie im gelben Abschnitt das Feld allein beherrschen; gelbes und braunes Pigment schließen sich also — einigermaßen — aus.

Die Markzellen enthalten im natürlichen Zustande des Haares neben den Pigmenthäufchen Luft; erst nach deren Verdrängung (z. B. durch den Canadabalsam im Präparat) läßt sich sicher beurteilen, welches Pigment vorliegt[1]. Das

[1] Man kann auch bei luftgefüllten Markzellen den gelbpigmentierten Anteil des Haarmarkes an dem starken weißlichen Aufleuchten der *Rinde* zwischen gekreuzten Polars leicht erkennen; diese ist nur im gelben Bereich frei von Melanin; an den braungefärbten Abschnitten wird die Erhellung der Rinde durch die hier vorhandenen Melaningranula merklich herabgesetzt.

Vertreiben der Luft wird durch Zerstückeln der Haare und Erwärmen des Präparates begünstigt, indem die sich ausdehnende Luft das Haar verläßt und der Balsam an den Schnittstellen bevorzugt eindringt. Abb. 3a zeigt ein Stück eines *gelben* Haarabschnittes, in der oberen Hälfte mit Luft gefüllt, in der unteren nach deren Verdrängung. Während oben nur die Interzellularen als helle Querstreifen in der dunklen Luftmasse hervortreten, zeigen sich unten die Häufchen der Granula; sie stoßen in Richtung der Haarachse an die ehemals dunklen mit Luft erfüllten Räume an, in deren Mitte die Keratinmäntel der Markzellen in Quergrenzen (*G*) sich berührten[1]. Ähnliche Bilder ergeben sich nach Luftverdrängung für die *melaninhaltigen* Zellen, indem auch hier die Granula in jeder Zelle zu einem Häufchen zusammengedrängt sind, das in der Achse des Haares beiderseits an Luft grenzt.

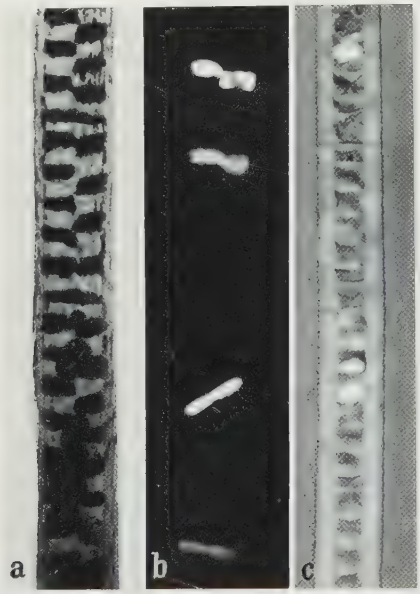

Abb. 4a u. b. *Funisciurus lemniscatus*, Haarabschnitt mit zweireihigem melaninpigmentiertem Markstrang. a gewöhnliches Licht; b Pol. +, 380:1: 4 kristalline Einlagerungen, die sich durch je 2 Nachbarzellen der beiden Reihen erstrecken; c *Heliosciurus poensis*, Haarabschnitt mit einreihigem gelbpigmentiertem Markstrang, 380:1, Pol. ×, drehbare $\lambda/16$-Glimmerplatte: mehrere den ganzen freien Raum der betreffenden Zellen erfüllende kristalline Einlagerungen

Die gelben Granula sind stärker lichtbrechend als Canadabalsam; bei Luftfüllung der sie enthaltenden Zellen sollte man zwischen gekreuzten Polars glauben, die Körnchen wären doppelbrechend; nach dem Vertreiben der Luft aber leuchten nur vereinzelte gelbe Granula schwach auf, die Erhellung der Granula in lufthaltigen Markzellen ist also darauf zu beziehen, daß an Luft reflektiertes Licht die gelben Körnchen beleuchtet.

Behandeln der Haare mit konzentrierter Schwefelsäure bringt die gelben Granula sogleich zum Verschwinden — im Gegensatz zu den sehr widerstandsfähigen Melaninkörnchen; jedoch bleibt eine gelbe diffuse Färbung (mit leicht bräunlichem Ton) eine Zeitlang zurück. Ebenso wirkt 10%ige wäßrige Kalilauge[2].

Die *kristallinen Einschlüsse* kommen sowohl in melanin- wie gelbpigmentierten Markzellen beider genannten Baumhörnchengattungen vor, jedoch nur spärlich, und zwar liegen sie neben den Pigmenthäufchen in den ehemals zum Teil von Luft eingenommenen, im Präparat mit Canadabalsam erfüllten Anteilen der Markzellen (vgl. Abb. 4a mit b). Abb. 4b zeigt bei *Funisciurus lemniscatus* in einem

[1] Nach Lühring (1928) soll beim Eichhörnchen die Luft im Markstrang *interzellular* liegen. Dieser Auffassung kann ich nach meinen Befunden bei Sciuriden, aber auch anderen Säugern mit ähnlich gebautem Markstrang — welche die Zellgrenzen meist deutlicher zeigen, so bei *Neomys fodiens* — nicht ganz zustimmen. Siehe auch Neumann (1937).

[2] Ob es sich bei den gelben Granulen um „Phaeomelanine" (Görnitz 1923) handelt, oder ob sie Beziehungen zu der gelben homogenen Pigmentmasse besitzen, die Lühring (1928) beim Eichhörnchen „zwischen einzelnen Markzellen" (siehe hierzu vorstehende Anmerkung 1) fand, bleibe dahingestellt.

melaninpigmentierten kurzen Abschnitt 4 aufleuchtende Einschlüsse. Alle bieten in der Flächenansicht des Haares stäbigen Umriß dar, sind aber, wie sich durch Fokusieren ergibt, plattenartige Gebilde, die dem Beschauer die Kante zeigen. Sie löschen nach der Kante aus und ihr Vorzeichen ist positiv zur Flächensenkrechten — was bei Fettgebilden solcher Art zu erwarten ist. Die kleine Brechzahl stimmt weitgehend mit der des Canadabalsam überein, so daß die Kristalle, mit der langen Kante nach der Schwingungsrichtung des allein eingeschalteten Polarisators orientiert, selbst bei enger Aperturblende nicht sichtbar sind (Abb. 4a); ihre große Brechzahl aber liegt beträchtlich über der dieses Einschlußmittels. Bemerkenswert ist, daß die Gebilde, wie der Vergleich von Abb. 4a mit b ergibt, durch je 2 Zellen sich erstrecken, die den beiden nebeneinanderliegenden Reihen der Markzellen angehören; diese Zellen müssen also durch eine Öffnung zusammenhängen. Sind die beiden Zellen insgesamt einigermaßen quer zum Haar orientiert, so stehen auch die Kristalle annähernd senkrecht zur Haarachse; sind sie aber in der Länge des Haares etwas gegeneinander verschoben, dann liegt der Kristall schief gegen die Haarachse (Abb. 4a, b).

In einem einreihigen Markstrang von *Heliosciurus poensis* fanden sich mehrere Zellen, deren jede — abgesehen vom gelben Pigment — fast ganz von einem Kristall erfüllt war (Abb. 4c). Einmal sah ich bei dieser Form im Spitzenabschnitt eines Haares ein längeres Kristallaggregat, das sich nicht mehr einzelnen Zellen zuweisen ließ.

Außerdem begegnet man kleinsten Teilchen des doppelbrechenden Materials. Bei *Funisciurus lemniscatus* traten solche an Haaren, die mit 10%iger Kalilauge behandelt waren (welche die Luft verdrängt hatte), vor allem in den gelben Abschnitten nach Zerstörung von deren Pigment reichlich als rundliche oder stäbige Körnchen hervor, die letzten negativ zur Länge.

Das geringe Material und die Kleinheit der doppelbrechenden Gebilde machten Versuche über ihr Verhalten gegenüber Erwärmen schwierig, zumal ich die wenigen guten Präparate nicht auf solche Weise zerstören wollte. Immerhin ergab sich, daß Haare, die ziemlich viele kleine doppelbrechende Körnchen darboten, nach dem Erwärmen nichts mehr davon erkennen ließen. —

Viel reichlicher als bei den im vorst nden behandelten afrikanischen Sciuriden finden sich fettartige Einlagerungen, wenn auch in etwas anderen Formen, bei dem einheimischen Eichhörnchen *Sciurus vulgaris*. Der Aufbau seiner Haare entspricht dem von *Funisciurus* und *Heliosciurus*, so daß sich näheres Eingehen darauf erübrigt. An die Stelle des gelbgrünen Pigmentes der genannten afrikanischen Baumhörnchen tritt bei *Sciurus vulgaris* ein rostrotes (Abb. 5a) mit ähnlichen Eigenschaften, z. B. rascher Löslichkeit in 10%iger Kalilauge. Jedoch bergen nicht nur die Zellen des Haarmarkes den rostroten Farbstoff, sondern auch die der Haarrinde freilich in geringerem Maße (Abb. 5a); er ist hier in Längsreihen geordnet entsprechend dem Verlauf der spindeligen Zellen, die ihn umschließen. Wie bei den behandelten afrikanischen Baumhörnchen das gelbgrüne Pigment, so ist das rostrote von *Sciurus vulgaris* nicht nur durch seine Farbe, sondern auch durch die Form seiner Granula scharf vom Melanin geschieden: sie sind rundlich, leicht kantig und von unterschiedlicher Größe (Abb. 5a). Zwischen gekreuzten Polars leuchtet dieses Pigment in luftgefüllten Markzellen mit prächtiger rostroter Farbe auf; jedoch sind die Granula ebensowenig wie bei dem

gelben Pigment doppelbrechend, sondern werden nur von Licht angestrahlt, das an den Lufteinschlüssen reflektiert wurde.

Ich untersuchte Grannen- und Wollhaare von Schwanz und Rumpf des Eichhörnchens. In den Grannenhaaren des Schwanzes wechselten rote und melaningefärbte Abschnitte mehrfach, in denen des Rumpfes ist meist nur ein mittlerer Abschnitt rot.

Leider ist die Verdrängung der Luft in den Haaren von *Sciurus vulgaris* wohl wegen der größeren Derbheit des Haarkleides schwerer zu erreichen, als bei den

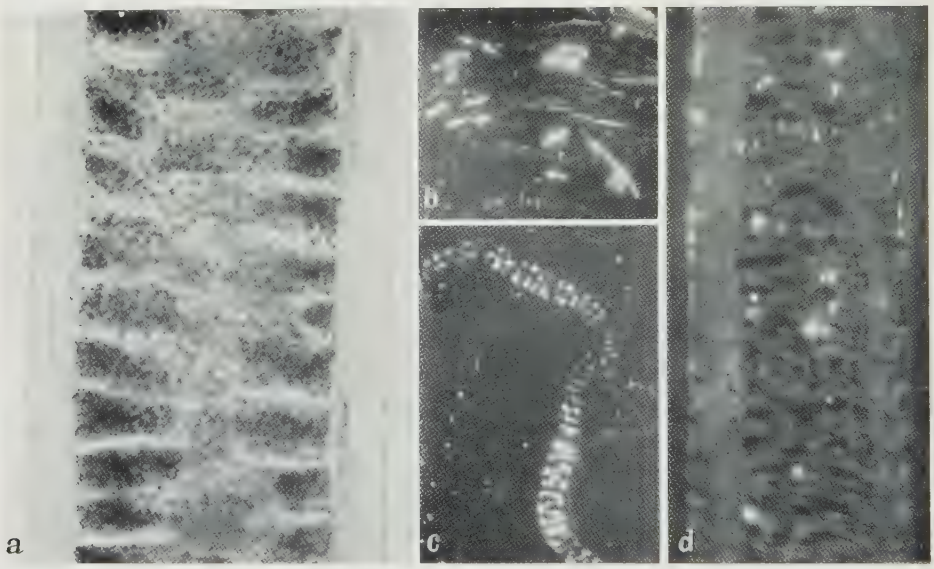

Abb. 5a—d. *Sciurus vulgaris*. a Rostbrauner Abschnitt eines Grannenhaares 830:1: Granula in den Markzellen, vereinzelt (rechts) auch in der Rinde; b Wollhaare mit Kalilauge behandelt, Pol. ×, 60:1: Abschnitte mit doppelbrechenden Einlagerungen hervortretend; c ebenso behandelt doppelbrechende Einlagerungen in aufeinanderfolgenden Markzellen eines Wollhaares, Pol. ×, 160:1; d melaninpigmentierter Abschnitt eines Grannenhaares ebenso behandelt, Pol. ×, 380:1, drehbare $\lambda/16$-Glimmerplatte: doppelbrechende Einlagerungen in den Markzellen

genannten afrikanischen Sciuriden. Nur nach Zerschneiden von Haaren dringt Balsam ein Stück weit in den Markstrang ein, während Erwärmen ganzer Haare im Präparat wenig wirksam ist. Daher bleibt man für die Untersuchung vor allem auf 10%ige Kalilauge angewiesen, die in einigen Minuten die Luft größtenteils aus den Haaren verdrängt und das rostrote Pigment löst.

In den Markzellen so vorbereiteter Grannen- wie Wollhaare treten an melanin- und an rostrotgefärbten Abschnitten stark doppelbrechende Einschlüsse hervor, jedoch nicht in allen Haaren, vielmehr sind manche so gut wie frei davon. Meist zeigen sich kleine Gebilde in vereinzelten Markzellen (Abb. 5d). Gelegentlich aber findet sich ein Dutzend oder mehr aufeinanderfolgender Markzellen mit umfangreichen Einschlüssen (Abb. 5b, c); diese stellen einen inneren Belag des Keratinmantels dar, der manchmal aus mehreren Teilchen besteht; alle aber sind feinbaulich so orientiert, daß die Schwingungsrichtung großer Brechzahl senkrecht zu der Unterlage steht. So kommen dann doppelbrechende Bildungen zustande, die an ein zur Hälfte ausgebildetes positives Polarisationskreuz

erinnern. Es tritt also hier die Neigung von Fetten zutage, sich mit der optischen Achse senkrecht zur Ansatzfläche zu orientieren (s. W. J. Schmidt 1959).

Erwärmt man Haare, in denen durch Kalilaugebehandlung die Fetteinlagerungen sichtbar gemacht wurden, über der Bunsenflamme, so schmelzen diese und nach dem Erkalten zeigen sich stark doppelbrechende negative Nadeln, oft in kugeligen Gebilden zusammengedrängt, die augenscheinlich zunächst als Fetttropfen vorlagen. Hier und da fanden sich auch kleine Sphäriten mit positivem Kreuz.

An Haaren, die mit einem Gemisch von Xylol und absolutem Alkohol zu gleichen Teilen extrahiert wurden, bringt Behandlung mit Kalilauge nur noch geringe Reste der doppelbrechenden Einschlüsse zum Vorschein.

Bei der Untersuchung der Haare von *Sciurus vulgaris* in Canadabalsam muß man sich vor Verwechslungen der Einschlüsse mit aufleuchtenden Anteilen der Keratinmäntel der Markzellen hüten. Diese sind wesentlich stärker doppelbrechend als bei den untersuchten afrikanischen Baumhörnchen und zeigen im optischen Durchschnitt an Polarisationskreuze erinnernde Auslöschungserscheinungen.

Gewiß werden sich in den Haaren weiterer Säuger kristalline Fetteinlagerungen der hier geschilderten Art nachweisen lassen. Eine systematische Erforschung dieser Verhältnisse unter Beachtung von Geschlecht, Lebensalter, Jahreszeit (Winter- und Sommerpelz), Ernährung, Biotop (z.B. Land- und Wassersäuger) wäre erwünscht; sie würde sich am Säugermaterial eines Museums oder Zoologischen Gartens mittels der Kalilaugebehandlung ohne besondere Mühe durchführen lassen.

Zusammenfassung

1. In *Markzellen* des Kopfhaares mancher *Menschen* finden sich nicht selten — wenn auch in wechselnder Menge für verschiedene Individuen und Lebensalter — kristalline schollige oder körnige, regellos gelagerte Einlagerungen fettartiger Stoffe; sie lösen sich in Lipoidsolventien, sind leicht schmelzbar und kristallisieren dann wieder aus. Die Lichtbrechung der Einlagerungen ist jener der der Umgebung so ähnlich, daß sie im gewöhnlichen Mikroskop nicht sicher wahrgenommen werden können. Cholesterin und Cholesterinester beteiligen sich nicht oder nur unwesentlich an den Ablagerungen; sie scheinen vielmehr aus Fettsäuren oder Neutralfetten zu bestehen, Stoffen, die wahrscheinlich bereits in den lebenden Markzellen abgelagert werden.

2. Gelegentlich kommen beim *Menschen* in der *Epidermicula* (sog. Cuticula) des Haares an vereinzelten Stellen doppelbrechende vermutlich fettartige Einlagerungen vor; sie liegen als feinste Lamellen zwischen 2 oder mehr Zellschüppchen und sind daher gleich diesen schräg zur Haaroberfläche gerichtet. Ihre Doppelbrechung ist stärker als die der Epidermicula; da ihre positive optische Achse senkrecht zur Lamellenebene steht, erscheint unter schwacher Vergrößerung das optische Vorzeichen der Epidermicula an diesen Stellen gegensätzlich zum gewöhnlichen.

3. Einzelne *Markzellen* der Haare der afrikanischen Sciuriden *Heliosciurus poensis* und *Funisciurus lemniscatus* umschließen in dem von den braunen oder gelben Pigmenthäufchen freigelassenen luftführenden Raum doppelbrechende plattige Kristalle einer fettartigen Masse, deren positive optische Achse senkrecht

zur Fläche steht. Solche Kristalle können sich auch durch 2 Zellen erstrecken, die nebeneinanderliegenden Markzellenreihen angehören. Ferner finden sich rundliche oder stäbige kleinere Teilchen derselben Masse in gleicher Lokalisation weithin über das Haar verstreut.

4. Die *Markzellen* von Grannen- und Wollhaaren des Eichhörnchens *(Sciurus vulgaris)* zeigen — in einreihigen Marksträngen an einem Dutzend Zellen und mehr hintereinander — einen inneren fettartigen Belag eines gewissen Bereiches ihrer Keratinmäntel, mit der positiven optischen Achse senkrecht zur Ansatzfläche.

5. Ferner werden einige Angaben über das *grünlichgelbe (Funisciurus, Heliosciurus)* und das *rostrote (Sciurus) Haarpigment* der untersuchten Baumhörnchen gemacht: die Granula sind rundlich-kantig, von wechselnder Größe, nicht doppelbrechend und im Gegensatz zu den stäbigen Melaninkörnchen der genannten Formen in Kalilauge leicht löslich.

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Aus dem Institut für Pathologische Anatomie der Medizinischen Akademie Poznan (Polen)

DIE FEINSTRUKTUR DES LUNGENGEWEBES NACH DEM BEGINN DER ATMUNG

Von

Janusz Groniowski und Wiktor Djaczenko

Mit 5 Textabbildungen

(Eingegangen am 10. November 1960)

Die fetale Lunge aus der Zeit des Überganges vom intrauterinem zum postnatalem Leben ist ein günstiges Objekt für die Erforschung der Korrelation zwischen Form und Funktion der Gewebekomponenten.

Die lichtmikroskopisch nachweisbaren Veränderungen in den Epithelzellen, die die Alveolargänge und zukünftigen Lungenalveolen in den letzten Wochen der Schwangerschaft auskleiden, sind bekannt, ebenso der Ausbau des Kapillarsystems des Lungenparenchyms in dieser Periode (s. Literaturverzeichnis). In Zusammenhang mit den neuen Lebensbedingungen sind Veränderungen auch der Ultrastruktur der einzelnen Zellen in den Lungen des Neugeborenen zu erwarten.

In einer im Jahre 1958 mündlich dargestellten elektronenmikroskopischen Untersuchung (Groniowski und Djaczenko 1960) beschäftigten wir uns mit der Ultrastruktur der Lunge eines reifen Rattenfetus, bei dem die Atemtätigkeit nicht einsetzte. Besondere Aufmerksamkeit wandten wir den Epithelien des Alveolarganges und der zukünftigen Lungenalveolen zu. In diesen Zellen fanden wir einen Ausbau des endoplasmatischen Retikulums, lamellenförmige Veränderungen der Mitochondrien, die von Schulz (1957, 1958) beschrieben wurden, und ziemlich viele lipoide Körper.

Die vorliegende Arbeit behandelt die Struktur des Blut-Luftweges der terminalen Atmungsmembran unter der Geburt. Gleichzeitig untersuchten wir die Epithelzellen, welche die Alveolargänge und die sich bildenden Lungenalveolen auskleiden.

Material und Methode

Zur Untersuchung gelangten die Lungen einer soeben geborenen 22 mm langen Ratte, die kurze Zeit geatmet hatte. Viele Lungenstückchen von verschiedenen Stellen wurden in einer 0,5% gepufferten Osmiumsäurelösung (p_H 7,35) fixiert, in Alkohol entwässert und in Butylmethakrylat eingebettet. Als Kontrollmaterial dienten Lungenstückchen eines Rattenfetus aus demselben Wurf. Die Ultradünnschnitte wurden mit einem Leitz-Rotationsmikrotom, das für Ultraschneiden adaptiert wurde, angefertigt. Zur elektronenmikroskopischen Untersuchung benutzten wir ein elektrostatisches D_2-Elektronenmikroskop von Carl Zeiss, Jena.

Ergebnisse

Die Lungen der neugeborenen Ratte werden sehr schnell entfaltet, doch war die Belüftung in unserem Falle sehr ungleichmäßig. Infolgedessen konnten wir verschiedene Stadien der Gestaltung der Atmungsmembran beobachten. Die Komponenten dieser Membran sind den entsprechenden Strukturen in der Lunge eines völlig reifen, schon längere Zeit atmenden Tieres sehr ähnlich

(Abb. 1). Sie bestehen aus folgenden Schichten: 1. Epithelschicht, 2. Mittelschicht, d. h. Basalmembran und 3. Endothelschicht.

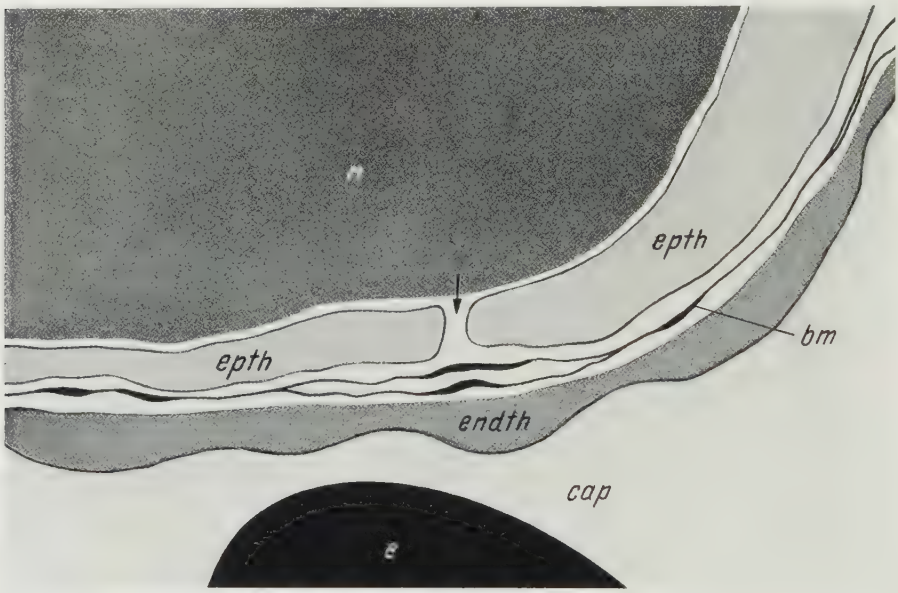

Abb. 1. *n* Epithelkern; Pfeil = Öffnung des perinukleären Raumes nach außen; *bm* Basalmembran; *epth* Zytoplasma der Epithelzelle; *endth* Endothelzelle; *cap* Kapillarlumen; *e* Erythrozyt

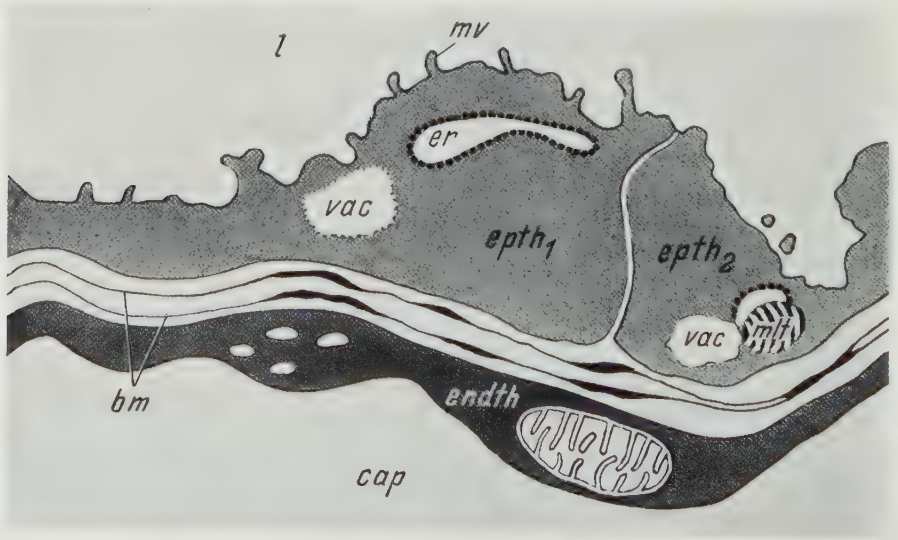

Abb. 2. Der Luft-Blutweg. $epth_1$, $epth_2$ Epithelzellen; *endth* Endothelzelle; *bm* Basalmembran; *cap* Kapillarlumen; *l* Alveolarlumen; *vac* Vakuole, durch Ausstoßung von Mitochondrientrümmern entstanden; *mv* Mikrovilli an der Oberfläche der Epithelzellen; *er* endoplasmatisches Retikulum; *mlt* lamellenförmige Transformation der Mitochondrien

Die *Epithelschicht* wird von einer dünnen Lage gedehnter Zellen gebildet, deren Grenzen an vielen Stellen deutlich auszumachen sind. Die abgeflachten Zellen tragen auf der Seite des Alveolarlumens zahlreiche Mikrovilli; diese sind

in der fetalen Lunge besonders zahlreich vorhanden (Abb. 2). Gegen die Mittelschicht (Basalmembran) ist die Epithellage in der Regel sehr deutlich abgegrenzt.

Die *Mittelschicht*, d. h. die Basalmembran, erscheint verschieden stark ausgebildet. In der Regel kann man an ihrer Epithelseite bei schwacher Vergrößerung (etwa 4000fach) eine osmiophile, parallel zur Schichtgrenze orientierte Lamelle erkennen. Eine zweite osmiophile Lamelle tritt parallel zur Endothelschicht in Erscheinung, doch fanden wir sie oft unterbrochen und weniger osmiophil als die an das Epithel angrenzende Lamelle. Die beiden Lamellen bilden

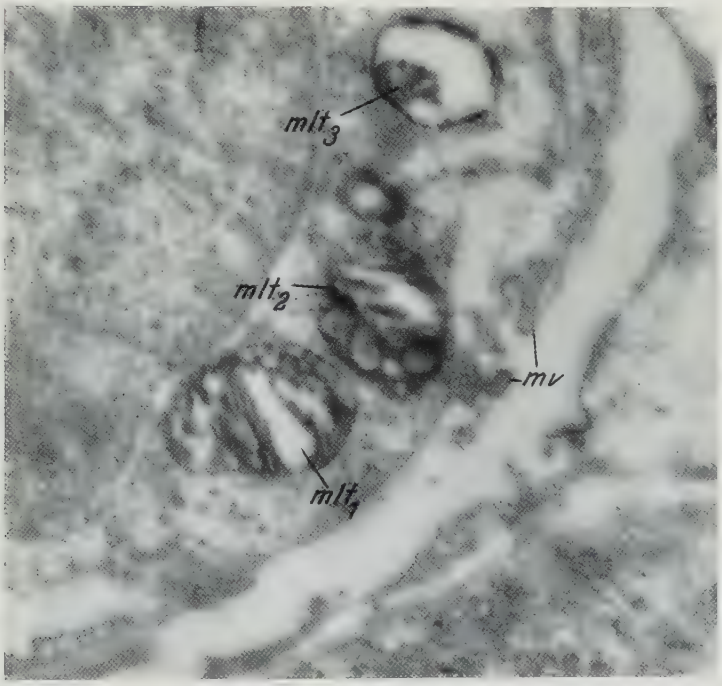

Abb. 3. Verschiedene Stufen der lamellenförmigen Transformation der Mitochondrien (*mlt 1, 2, 3*) in der Epithelzelle. *mv* Mikrovilli. Vergr. 31000 ×

die Oberfläche der Basalmembran, die sich mithin in drei Zonen verschiedener Dichte gliedert (vgl. Abb. 2). Die Grenze zwischen Endothel und Basalmembran ist stets deutlich sichtbar.

Die *Gestalt der Epithelzellen*, welche die Alveolargänge und die sich bildenden Alveolen auskleiden, hängt von der Belüftung des Lungenparenchyms ab. Ihre Formenreihe erstreckt sich von den kubischen Elementen in den schwach belüfteten Regionen bis zu den abgeflachten Zellen in den voll belüfteten Bezirken.

Die Kerne der Epithelzellen werden durch die bekannte Doppelmembran gegen das Zytoplasma abgegrenzt (Abb. 1). Mitunter findet sich zwischen beiden Membranen eine Erweiterung. Unter besonders günstigen Bedingungen, d. h. dann, wenn der Zellkern ausgesprochen exzentrisch im Zelleibe liegt, kann man den unmittelbaren Zusammenhang des perinukleären Spaltes mit dem extrazellulären Raum feststellen (Abb. 1). Dieser Raum wird dann auf der einen Seite durch eine Zytoplasmamembran, auf der anderen Seite durch eine parallel zu

ihr verlaufende osmiophile Schicht der Basalmembran begrenzt. Eine oder zwei herdförmige Verdichtungen im Kernraum entsprechen den Nukleolen.

Die Zelloberfläche der kubischen Epithelzellen in den wenig belüfteten oder unbelüfteten Regionen der Lunge ist mit einzelnen, seltener mit Gruppen kurzer, schmaler *Mikrovilli* besetzt. Invaginationen des Plasmalemms, die in der unbelüfteten Lunge der Ratte sehr häufig zu beobachten sind, waren in unserem Material sehr selten zu sehen. Ferner ist das *endoplasmatische Retikulum* schwach entwickelt bzw. an manchen Stellen kaum erkennbar. In den kubischen Epithelzellen nimmt es teils die Umgebung des Zellkernes ein, teils liegt es in der Peripherie

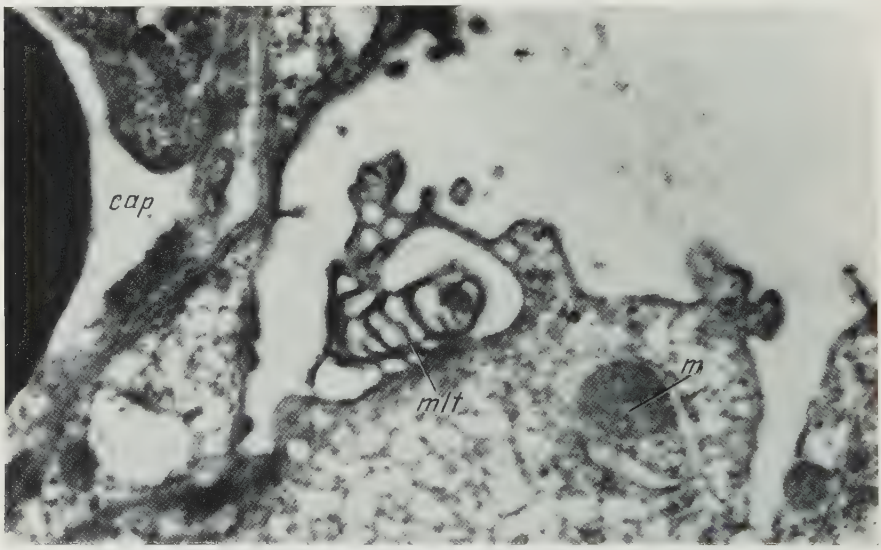

Abb. 4. Lamellenförmig umgewandeltes Mitochondrium (*mlt*) in einer Zytoplasma-Knospe. Normales Mitochondrium (*m*), Kapillarlumen (*cap*). Vergr. 24 000 ×

der Zelle in der Nachbarschaft der Mitochondrien. Die Membranen des endoplasmatischen Retikulums, das in Form von Kanälchen und Bläschen auftritt, sind in der Regel mit Körnchen besetzt (Palade 1956). In den Zellen der unbeatmeten Lunge dagegen liegt das endoplasmatische Retikulum mit granulierten Oberflächen in den zentralen Partien der Zelle in Kernnähe, während man in der Peripherie nur glatte Profile des Retikulums beobachten kann. Von osmiophilen Membranen begrenzte Vakuolen, die in der unbeatmeten Lunge häufig zu finden sind, waren in unserem Material nicht sichtbar.

Die *Mitochondrien* der Epithelzellen liegen teils nahe dem Zellkern, teils nahe dem lumenwärtigen Plasmalemm. Die zentral gelegenen Mitochondrien besitzen normale Bauweise. Dagegen sind die Mitochondrien in der Nähe der Zellmembran meistenteils verändert. Sie zeigen verschiedene Stadien der *lamellären Transformation*, wie sie von Schulz (1958) beschrieben wurde. Im einzelnen beobachteten wir die Ausweitung der Bezirke zwischen den Cristae mitochondriales; hier wurden auch vesikuläre Aufhellungen festgestellt (Abb. 3). Ferner scheint es zum Untergang der Matrix mit Durchbrechung der äußeren Membran zu kommen. Schließlich findet man zerfallende Mitochondrien mit intensiv osmiophilen Membranfragmenten. Sehr stark zerbröckelnde Mitochondrien liegen in einem

Zytoplasma von geringer Dichte. Die lamelläre Transformation der Mitochondrien wurde sowohl in unbeatmetem Lungengewebe als auch sehr häufig in der Lunge nach den ersten Atemzügen beobachtet. Außerdem bemerkt man eine Veränderung, die in der unbeatmeten Lunge nur selten, in der belüfteten des Neugeborenen sehr häufig auftritt, nämlich das Vorkommen lamellär trans-

Abb. 5. Viele lamellenförmig transformierte Mitochondrien (*mr*) im Alveolarlumen (*l*). Normale Mitochondrien (*m*). Kapillarlumen (*cap*). Vergr. 12500 ×

formierter Mitochondrien oder Mitochondrientrümmer *im Lumen der Alveole* (Abb. 4) oder des Alveolenganges. In solchen Fällen werden auch andere Zytoplasmateile mit den Mitochondrien ausgestoßen, nämlich Abschnitte des endoplasmatischen Retikulums (rauhe Profile). Die Ausstoßung der Mitochondrien scheint sich folgendermaßen abzuspielen: Das Zytoplasma bildet knospenartige, transformierte Mitochondrien enthaltende Gebilde (Abb. 5). Von derartigen vorgebuchteten Teilen des Zelleibes werden die transformierten Mitochondrien ausgestoßen. In anderen Fällen scheint die Abgabe der Mitochondrien auch ohne Knospenbildungen vonstatten zu gehen, da man auf zahlreiche Räume im Zytoplasma nahe der Alveolenlichtung stößt, in denen sich die veränderten Mitochondrien befinden; diese Räume kann man auch im unbeatmeten Lungengewebe feststellen.

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THE ULTRASTRUCTURE OF CHOANOCYTE COLLARS IN *SPONGILLA LACUSTRIS* (L.)

By

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With 9 Figures in the Text

(Received November 3, 1960)

Introduction

The freshwater sponge *Spongilla lacustris* (L.) is composed of four main cell types (Brondsted 1936). One of these are the choanocytes that form the flagellate chambers. They are characterized by a lamp-glass shaped "collar" which surrounds the proximal part of a long flagellum. The body of the cell is about 6 μ long, 3—4 μ broad, and provided with a small nucleus without nucleolus.

The collar of the choanocytes has generally been regarded as a cytoplasmic tube, possibly formed by a rolled-up sheet of cytoplasm, thus Hentschel (1923 to 1925) writes as follows: „Die Geißelzellen oder Choanocyten der Kalkschwämme bestehen aus einem Körper von veränderlicher Gestalt, einem im allgemeinen zylindrischen, doch je nach dem Kontraktionszustande sehr veränderlichen Kragen, von dem neuerdings behauptet wird, er sei keine eigentliche Röhre, sondern ein eingerolltes Blatt, und der daraus hervorragenden Geißel." Other authors have expressed a similar view, so Ankel (1950) who writes about *Spongilla:* . — der aus einer äußerst zarten Plasmahaut bestehenden Kragen". Kilian (1952) expresses himself much in the same way about *Ephydatia fluviatilis:* „Der Kragen besteht aus einer Plasmawand von äußerst geringer Dicke. Er kann völlig eingezogen und in verschiedenem Maße entfaltet werden. Ich fand ihn immer homogen und durchsichtig." A somewhat different opinion, after which the cytoplasmic collar is supported by fine fibrils, finds its foremost representative in Farkas (1929). Kilian (1954) now has changed his original view after an electron microscopic investigation of whole choanocytes and is now of the same opinion as Farkas.

As these authors have mainly concerned themselves with light microscopical investigations of the collar, it was to be expected that the use of the electron microscope might throw light on the problem.

A paper on the fine structure of the collar found in the choanoflagellates (Craspedomonadaceae) is in preparation.

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Materials and methods

Both full-grown sponges collected in the autumn, and quite young ones from single germinating gemmules were investigated. The gemmules were sampled in February and stored in water at 2—4° C in the icebox. The water was changed with few days interval. For use the gemmules were germinated on polyethylene plastic in water of room temperature, and fixed in situ. The employed gemmules were germinated 3—8 days before fixation.

Both types of material were fixed for $1^1/_2$ hours at 4° C in buffered osmium tetroxide at p_H 6,2 and embedded in methacrylate (1 part of methylmethacrylate to 9 parts of butylmethacrylate). Sections were cut on a SJÖSTRAND ultramicrotome, adjusted to cut sections about 500 Å thick. In one case it was possible to calculate a thickness of 320 Å of the sections by means of obliquely cut structures, according to the method described by GETTNER and ORNSTEIN (1956).

The sections were stretched with vapour of xylene and transferred to formvar-coated grids. Part of them were examined without further treatment, but most of the sections were stained with a 1% solution of potassium permanganate after the method of LAWN (1960). They were stained for $^1/_2$ to 2 hours. Best results were obtained by staining for 2 hours and rinsing in citric acid solution (1 drop of 5% citric acid in 1 ml water) for 1 minute. This method provides a strong general staining of the cells, by means of which focussing is facilitated, particularly at the high magnifications, and certain structures, such as the unit membrane structure of the cell membrane (ROBERTSON 1959) become easily discernible.

A few sections were also stained with uranylacetate after WATSON (1958).

The sections were examined in a RCA EMU 2B electron microscope at magnifications from 1200—24000. The order of magnitude of the highest resolution obtained was 20—30 Å.

Observations

I. Introductory description

The choanocytes can be recognized by their characteristic nucleus which contains electron dense particles about 5000 Å in diameter (Figs. 1, 3). The nuclear membrane is on the inner side accompanied by a 800—1800 Å thick electron dense layer. Lower magnifications of cross sections show that the "collar" consists of "fibrils" arranged circularly with a regular spacing of about 1300 Å (Fig. 1). The number is varying, 6 counts gave 1 "collar" consisting of 31, 2 of 32, 2 of 33, and 1 of 34 fibrils. However, it is difficult to count the fibrils, because the various "collars" in a flagellate chamber often adhere so closely that is hard to tell to which "collar" the different fibrils belong.

In the middle of the circular pattern formed by the fibrils lies the flagellum (Figs. 1 and 2), which will be treated in a later section.

The shape of the single fibrils is variable (Fig. 2). Their diameter is 1000 to 2000 Å. In different sections it is seen, that the distance between the fibrils is varying with the height above the "bottom" of the collar, from about 3000 Å to less than 1000 Å, at times even contact.

At the base of the collar it is seen, even at low magnification, that the limiting membrane of the fibrils passes directly into the cell membrane of the choanocyte. This is observed both in longitudinal sections (Figs. 3 and 4), and in such obliquely cut cross sections, where the collar is hit at the base. Accordingly the fibrils may be regarded as a kind of pseudopodia. In the bottom of the collar there is a homogeneous, slightly electron dense layer (Figs. 1 and 3), probably mucus. These features are all common to both young and older sponges, and may be observed at low magnification.

II. The fine structure of the collar fibrils

On high resolution micrographs, the individual collar fibrils are limited by a 3-layered membrane consisting of 2 dark layers with a lighter middle layer between (Figs. 5 and 6a). Peak to peak of the dark layers the membrane is about 50 Å. This membrane is of the same appearance as the cell membrane surround-

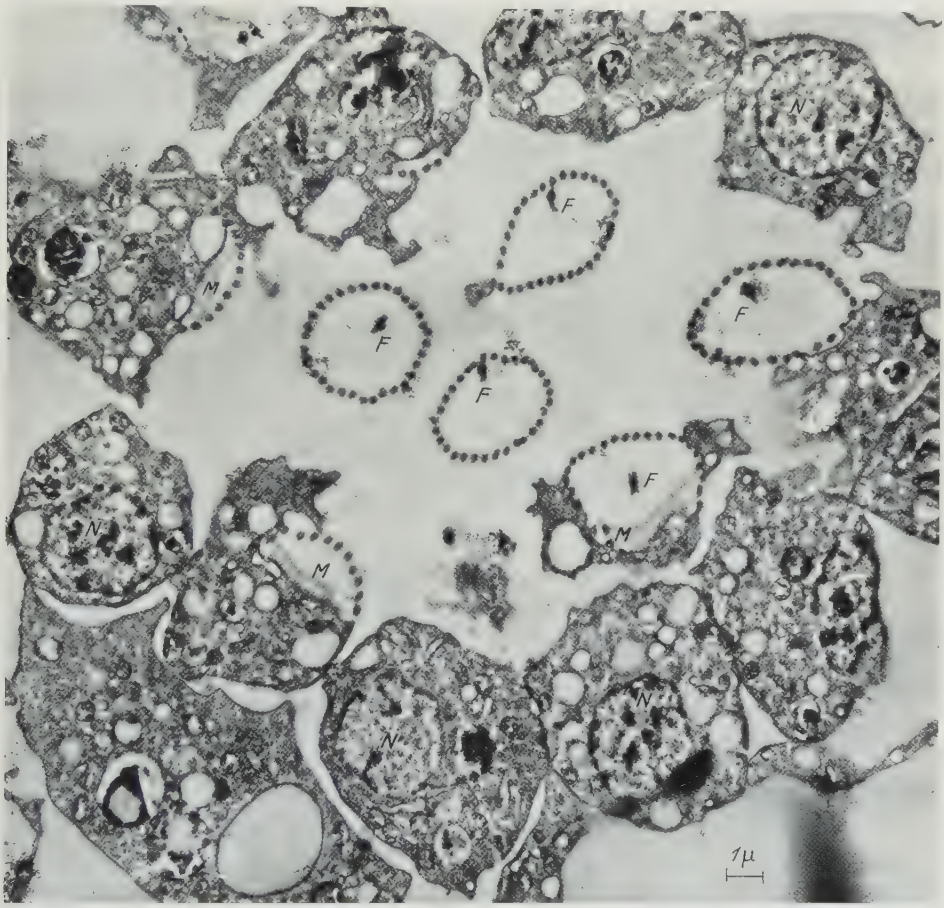

Fig. 1. Section of a whole flagellate chamber. Collars are sectioned both transversal and obliquely, the collar fibrils are evident. The granular appearance of the choanocyte nuclei (*N*) is seen. *F* flagella; *M* probably mucus in the bottom of the collar. Young sponge from gemmule germinated 8 days. Section stained with $KMnO_4$. ×4900

ing the choanocyte. The structure is identical with the "unit membrane" described by authors who have investigated the cell membrane at high resolution, for instance Robertson (1959) and Mercer and Shaeffer (1960).

Against the inner side of the membrane of the fibrils there very often is an electron dense layer (Fig. 5), which when observed in cross sections is ring-shaped and surrounds a lighter central part of the fibril. The thickness of this layer is about 350 Å.

Even at medium magnifications one may at times in cross sections suspect a connecting shadow between some of the fibrils in a collar (Fig. 2). At high magnifi-

cation of unstained sections or sections stained in uranylacetate it is still only indistinctly seen, that some material may connect the fibrils. However, if the sections are stained with potassium permanganate, it is clearly observed, that the collar fibrils are linked together by several parallel microfibrils (Figs. 6a and b, and 7). These are extremely thin, about 40 Å, and in the best micrographs seem to have a beaded appearance (Fig. 6b). The microfibrils are also seen in

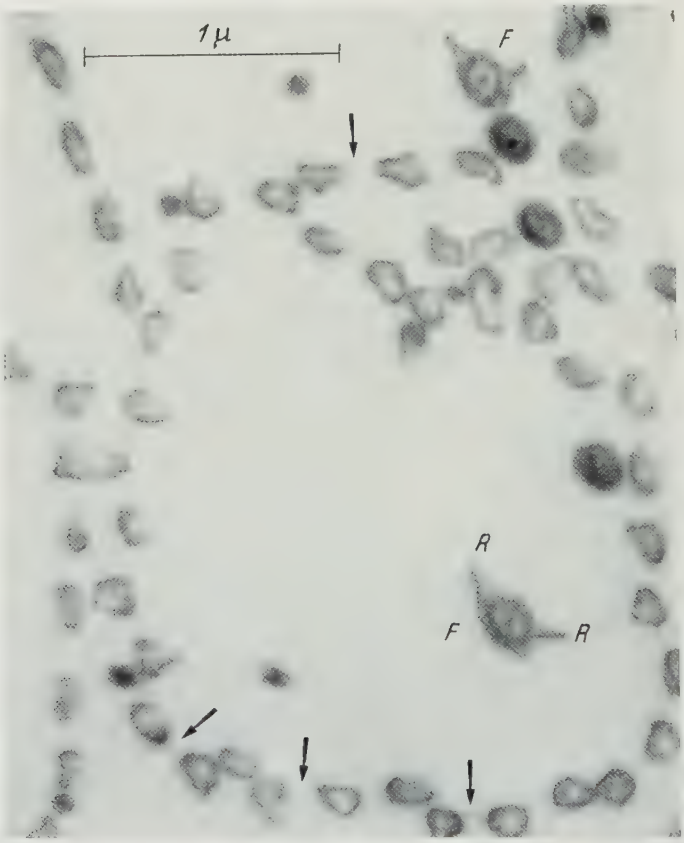

Fig. 2. Cross section through a single collar. The variable shape of the collar fibrils is illustrated. Parts of adjoining collars are seen. Two flagella (*F*) are seen having the typical internal structure; *R* projecting ridges of the flagellum. At the arrows some connecting material may be suspected between the collar fibrils. Adult sponge, unstained section. ×33280

longitudinal sections of the collar fibrils (Figs. 4 and 5), where they can be seen to be irregularly distributed along the length of the fibrils (Fig. 4), at intervals they are even missing. It seems that the microfibrils are not found at the same height on all of the fibrils of a collar, on some cross sections they may be seen between some of the collar fibrils but not between others.

In certain sections it is clearly realized, that the microfibrils arise from the surface of the limiting membrane of the collar fibrils, and that they do not penetrate the interior of the fibril (Fig. 5 and 6a). In some places micro-fibrils are also situated in a direction radially to the collar (Fig. 7).

III. The flagellum

Although the principal topic of this paper is the structure of the choanocyte collar, a brief comment on the flagellum may be given.

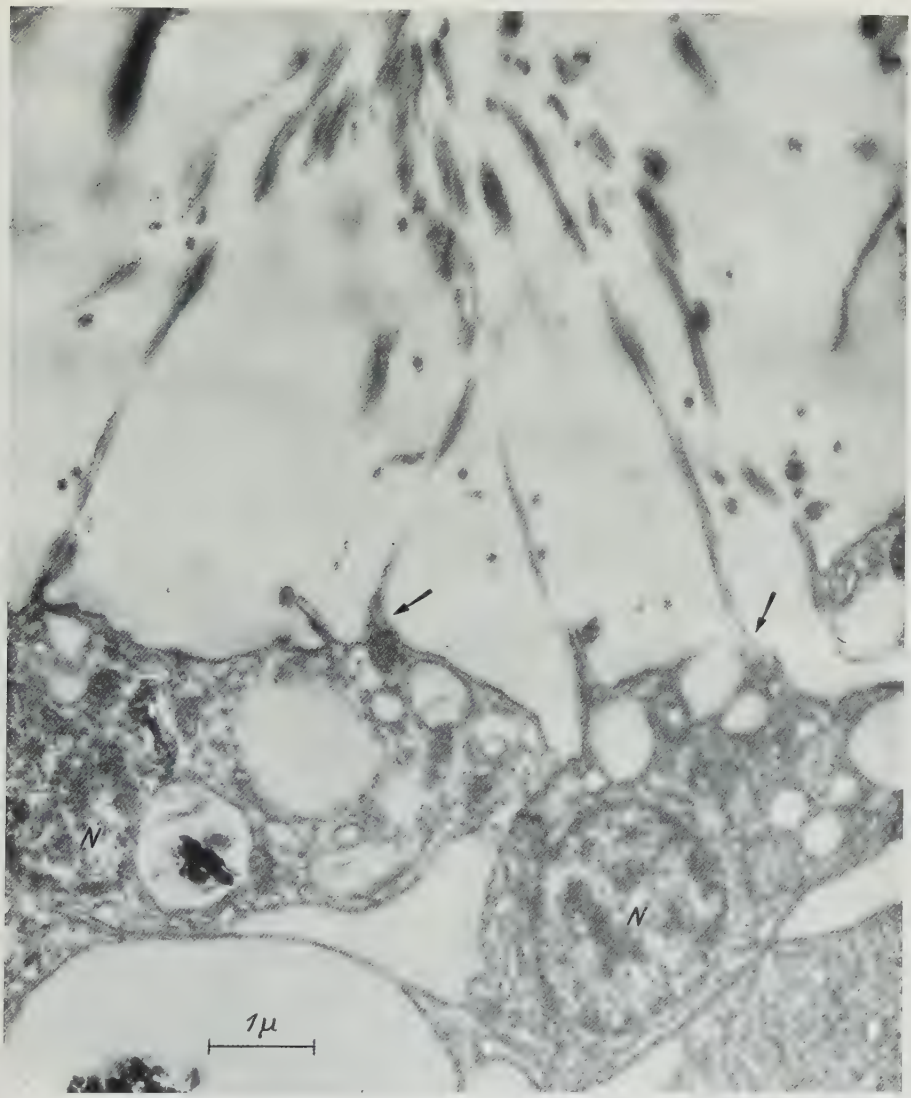

Fig. 3. Longitudinal section through two choanocytes and their collars. At the arrows it is seen how the cell membrane and the membrane of the collar fibril passes into one another. *N* nucleus. Adult sponge, unstained section ×12764

Even at medium magnification (Fig. 2) the construction that is well-known from other flagella is seen: inside the flagellum, which is about 2000 Å in diameter, is a central pair of fibrils surrounded by nine outer pairs of fibrils. Higher magnifications (Fig. 8) show, that the fibrils are cylinders, invested by a 40 Å thick outer membrane. The two central fibrils are each 200 Å in diameter. The

outer fibrils are 250 Å broad each, and their joint width is 330 Å. These dimensions are about the same as those reported for other flagella and cilia for instance by Rhodin and Dalhamn (1956) for the cilia in the trachea of rat, and by Gibbons and Grimstone (1960) for the flagella of certain flagellates. In some cases there are indications in the outer fibrils of the "arms" (Fig. 8), which are

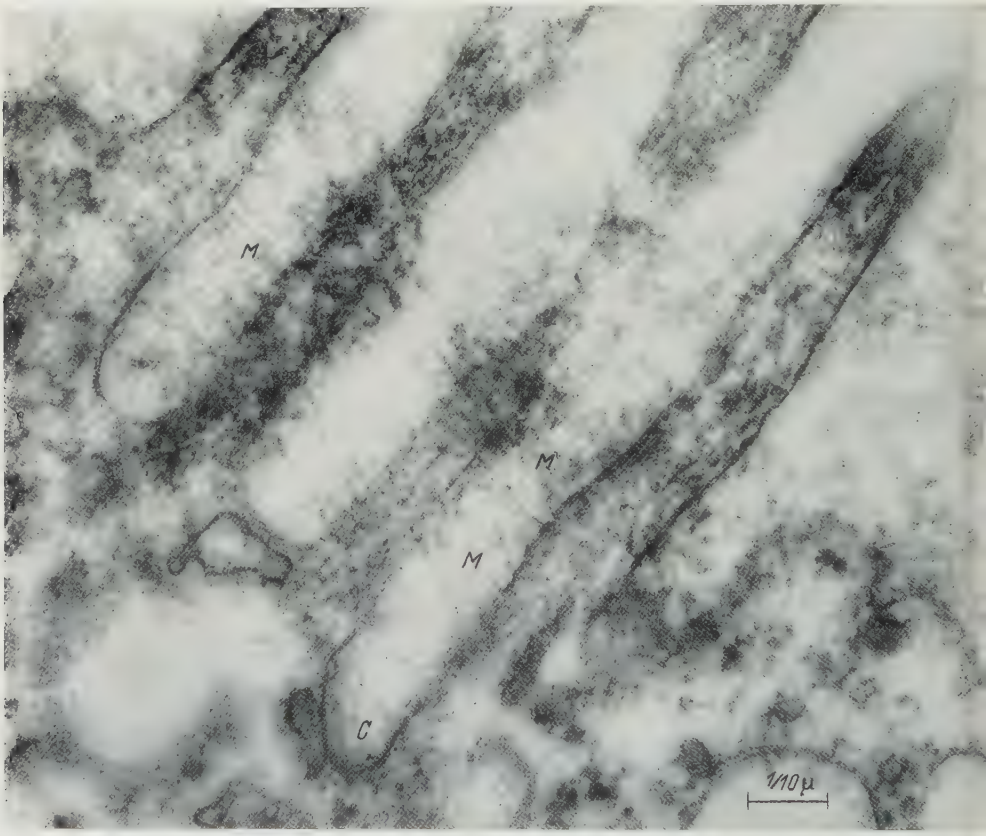

Fig. 4. Longitudinal section of a number of collar fibrils at the base of the collar. The pseudopodic structure is evident. At *C* the cell membrane, partly resolved as "unit membrane", is seen to communicate with the membrane of the collar fibrils. The microfibrils connecting the collar fibrils are shown, their irregular distribution is illustrated; the highest concentrations are found at *M*. Gemmule germinated 7 days, section stained with $KMnO_4$. ×105000

mentioned by Afzelius (1959) in the tail of echinoderm sperm and by Gibbons and Grimstone (1960).

The membrane of the flagellum often shows protuding ridges along the flagellum (Fig. 2).

Proximally the flagellum arises from a structure consisting of 9 fibrils forming a circle and inclined inwards, that is to say they form an angle to the tangent through the middle of the fibril (Fig. 9). These fibrils are about 500 by 200 Å. There are indications of their being triple, and on Fig. 9 they are seen to be surrounded by a 700 Å broad zone of electron dense material. This corresponds to the "basal plate" of Gibbons and Grimstone (1960), and the whole formation is

Fig. 5. Obliquely longitudinal section of a single collar fibril. The "unit membrane" of the fibril is clearly seen (*U*). At *M* the microfibrils are seen to arise from the surface of the membrane. The dark outer layer of the interior of the fibrils is seen just inside the membrane. Gemmule germinated 6 days, section stained with $KMnO_4$. ×280000

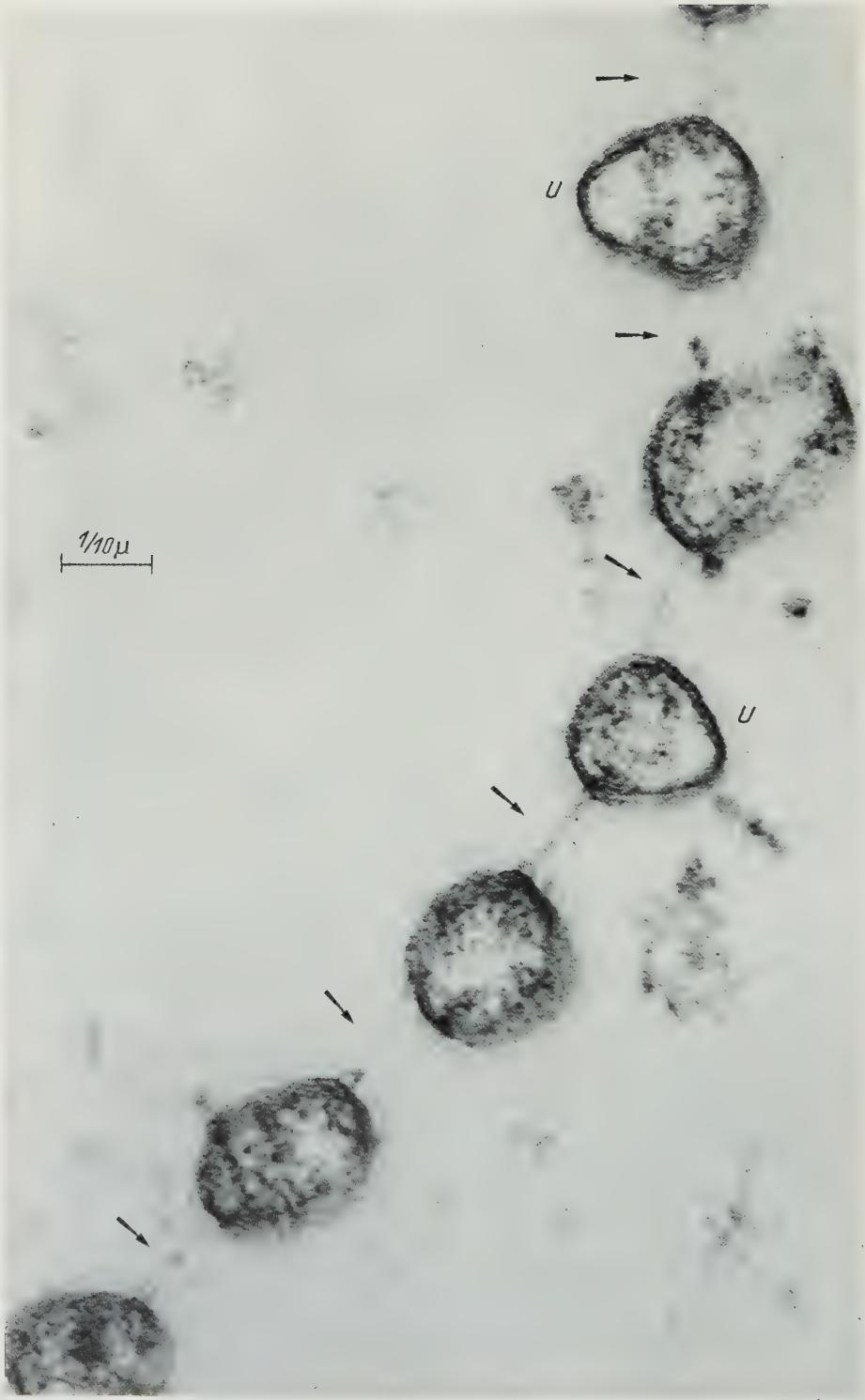

Fig. 6a

Fig. 6b

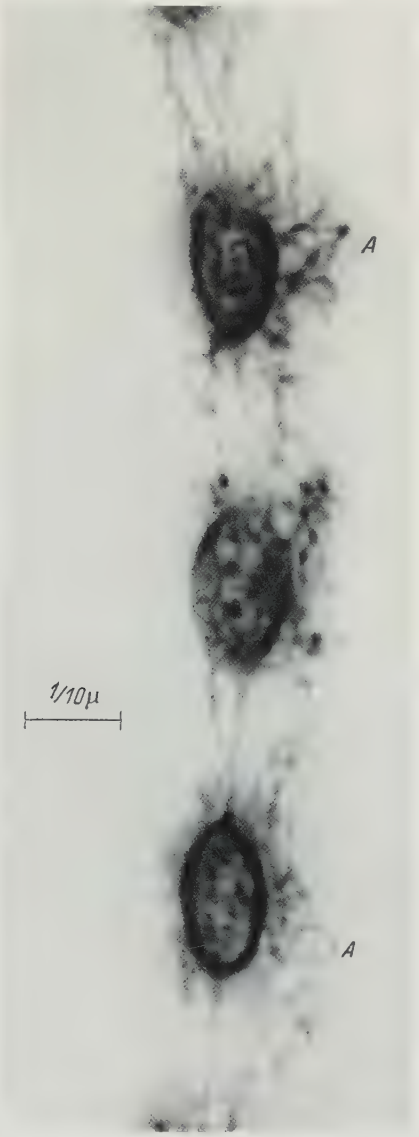

Fig. 7

Fig. 6a. Cross section through part of a collar. At the arrows the microfibrils, that connect the individual collar fibrils are seen. At *U* the "unit membrane" of the collar fibrils is shown. Gemmule germinated 6 days, section stained with $KMnO_4$. ×126500

Fig. 6b. Further enlargement of Fig. 6a. The microfibrils are clearly seen, at *B* their beaded appearance is evident. ×265000

Fig. 7. Cross section through a part of the collar with a large number of microfibrils, corresponding to *M* in Fig. 4. At *A* the microfibrils are also situated radially to the collar. Gemmule germinated 7 days, section stained with $KMnO_4$. ×125000

of the same structure as the "basal body" in *Holomastigoides*, but the fibrils are inclined to the opposite side, they are oriented "counter clockwise".

Around the basal body are found some 400 Å broad ribbons, sometimes cross-striated. They serve possibly to fasten the flagellum and the basal body to the cell.

The basal body is very similar to the centriole described by DE HARVEN and BERNHARD (1956) in the cells of certain vertebrates. The reason for flagella and cilia having 9 + 1 pairs of fibrils have recently been discussed by SERRA (1960).

Fig. 8. The flagellum cut transversely, showing the 9 double outer cylinders surrounding the two in the middle of the flagellum. The arrow points to a structure probably identical with the "arms" of AFZELIUS. Gemmule germinated 8 days, section stained with $KMnO_4$. × 240 000

Discussion

These observations show that the choanocyte collar in *Spongilla lacustris* (L.) is not a homogeneous structure. It consists of circularly arranged long, thin projections from the cell. They are almost parallel, and together they form, what in the light microscope is seen as a hyaline, structureless collar, because both the individual projections and their mutual distance are of such small order of size, that they cannot be resolved by the light microscope. The projections, in the foregoing called collar fibrils, are invested by a typical cell membrane, passing directly into the membrane surrounding the choanocyte, and thus it seems justified to describe them as very long and thin pseudopodia. This interpretion also explains why they are so variable in size and shape. Such pseudopodia would have difficulty in forming a regular collar by their rigidity alone, and they are supported by being connected through very delicate microfibrils.

These microfibrils that connect the pseudopodia to each other, are evidently of great importance. Only by means of these the collar acquires its stability and rigidity which is important for its function and which is one of the reasons why previous investigators believed that it was a solid structure.

The fact that the collar is composed of pseudopodia also explains how it can be drawn into the body of the cell, when this is influenced by some kind of irritation as described by several authors, for instance KILIAN (1952) for *Ephydatia fluviatilis* and DE LAUBENFELS (1932) for *Iotrochota birotula*. Complete withdrawal of the collar nearly always results when the sponge is squeezed through

cloth in order to dissociate the cells (Brøndsted 1936; de Laubenfels 1932). Whether the delicate microfibrils, that connect the pseudopodia, are lost by partial withdrawal of the collar or not, is not known.

From the foregoing observations it appears that Farkas' (1929) and Kilian's (1954) concept of the collar as a membraneous cytoplasmic formation with supporting fibrils is wrong, for no connecting membrane and no cytoplasm around the pseudopodia are found. The fibrils mentioned by Farkas are in his illustrations shown too coarse to represent the pseudopodia, most probably they are artifacts arising from the fixation of the collar. Farkas is of the opinion, that the fibrils continue far down into the body of the choanocyte. This is not the case with the pseudopodia. Still Farkas' views of the structure of the collar

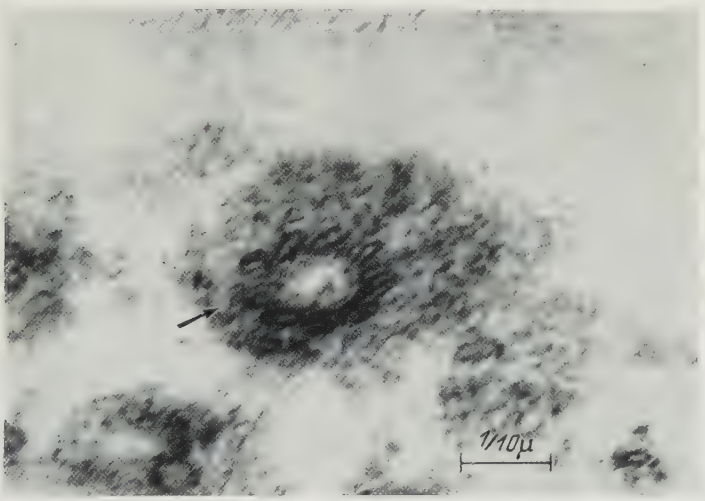

Fig. 9. The "basal body" with "basal plate". At the arrow one of the fibrils seems to be triple. Gemmule germinated 8 days, section stained in $KMnO_4$. ×116800

is more correct than the other previously proposed theories, even if he insists that the collar is a solid formation, because his fibrils are embedded in a sheet of hyaline cytoplasm. As for Kilian's (1954) finding with the electron microscope that the collar is composed of cytoplasm and fibrils, this is probably an artifact due to the employment of conventional light microscopic fixatives.

A note on the function of the collar

It has been known for a long time, that the function of the choanocytes partly is to produce a flow of water through the sponge and partly to filter off small particles, serving as food for the sponge (van Trigt 1919; Kilian 1952 and 1954).

It has been noticed by the former author, that the small particles entering the flagellate chamber with the water are trapped on the outside of the collar and from here transported into the cell. This investigator has observed, that when the food particles reach the level of the collar they suddenly deviate and stick to the collar. How this goes on does not seem to have been understood correctly previously. Kilian (1952 and 1954) is of the opinion that the most important function of the collar is to give the flow of water a definite direction,

viz. towards the apopyle, by so to speak steering the flagellum. He comments further (KILIAN 1952): „Auf keinen Fall kann daran gedacht werden, daß Wasser aus den Kragenröhren selbst ausströmt. Innerhalb des Kragens konnten wir nie irgendwelche Strömungserscheinungen beobachten, und ebensowenig sind dort Partikel zu finden. Schon aus den Dimensionen des Kragens muß geschlossen werden, daß das Wasser in seinem Innern infolge der Randkräfte weitgehend eine „zähe Flüssigkeit" ist und keinen Raum oder Möglichkeit für zwei gegenläufige Strömungen bietet, die ja angenommen werden müßten, wenn durch die Geißelbewegung aus dem Kragen Wasser strömen sollte. Eine Diffusion von Wasser durch die Kragenmembran dürfte freilich möglich sein, da diese nur die Dicke von einigen Eiweißmolekülen haben kann."

This statement is caused by the opinion, that the collar is a homogeneous membrane. As shown by electron microscopic observations this is not the case, and the following hypothesis is proposed to explain the function of the collar: 1. The flagellum gives rise to a flow of water that leaves the collar at the top. 2. More water is sucked in between the pseudopodia of the collar. 3. In the network formed by the pseudopodia and connecting microfibrils all particles having a diameter more than about 2000 Å are trapped.

This hypothesis explains, why KILIAN (1952) did not see any particles inside the collar, and why VAN TRIGT (1919) observed the particles suddenly deviate towards the collar.

Summary

This paper is a description of the ultrastructure of the collar of the choanocytes in the freshwater sponge *Spongilla lacustris* (L.). Both young and older sponges were examined. Previously the prevalent opinion has been that the collar is a homogeneous, membraneous "funnel". The present investigation shows that the collar actually is formed by a great number of fibrils, whose fine structure has been examined by appropiate staining of ultrathin sections. It is shown that these fibrils probably are very thin pseudopodia and further how these pseudopodia are held together to form a collar. This is effected by a large number of very delicate microfibrils that connect the individual pseudopodia thus forming a network together with the pseudopodia. By this method the collar attains a rather high degree of stability.

The flagellum was also examined. Its structure is the well-known (9 + 1 pairs of fibrils). The basal body has a construction similar to that of a centriole.

The function of the collar is discussed in relation to its ultrastructure and it is proposed that it is a filtering organ. It is demonstrated that this hypothesis is able to account for previous observations by other authors on the feeding of sponges.

Postscript

After having finished this paper, the author became acquainted with the papers by RASMONT, BOUILLON, CASTIAUX and VANDERMEERSCHE (Compt. Rend. Acad. des Sci., t. 245, p. 1571—1574, séance du 28 octobre 1957) and RASMONT [Ann. Sci. Nat. Zool., XII. ser., 253—262 (1959)] dealing with the submicroscopic structure of the collar of *Ephydatia fluviatilis*. Their micrographs of ultrathin sections show beautifully, that the collar is built of a great number of free fibrils regularly spaced. However, these authors have not been able to demonstrate the microfibrils connecting the collar fibrils and the unit membrane limiting the latter.

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HISTOLOGISCHE UNTERSUCHUNGEN ÜBER DIE PHENOLROTAUSSCHEIDUNG WÄHREND DER KOMPENSATORISCHEN HYPERTROPHIE DER RATTENNIERE*

Von
Gerhard Wölker

Mit 7 Textabbildungen

(Eingegangen am 1. Dezember 1960)

Die Niere kann harnpflichtige Stoffe nicht nur glomerulär, sondern zusätzlich auch tubulär eliminieren. In zahlreichen Untersuchungen wurde dieser doppelte Ausscheidungsmodus für bestimmte Substanzen, wie Phenolrot, p-Aminohippursäure, Penicillin und Röntgenkontrastmittel immer wieder beobachtet. Sehr früh erkannte man, daß Phenolrot bei allen untersuchten Tieren (Fischen, Amphibien, Sauropsiden und Säugetieren) auch durch das Tubulussystem ausgeschieden wird (Marshall u. Vickers 1923, Edwards u. Marshall 1924, Bieter u. Hirschfelder 1924, Smith 1939). Chambers und Cameron (1932) waren die ersten, die an ausgeschnittenen Tubulusstücken der Hühnchenniere in der Gewebekultur feststellen konnten, daß nur die Hauptstücke fähig waren, das der Zuchtlösung zugesetzte Phenolrot im Lumen zu konzentrieren. Alle anderen Nephronabschnitte und die Sammelrohre waren dazu nicht in der Lage. Die Clearance-Untersuchungen von Shannon (1935) brachten eine weitere bedeutsame Erkenntnis: die glomeruläre Ausscheidungsrate für Phenolrot ist der Farbstoffkonzentration im Blutplasma proportional, während die Hauptstücke nur eine beschränkte Ausscheidungskapazität besitzen. Diese kann selbst bei höchster Farbstoffkonzentration im Blut nicht über ein bestimmtes Maximum gesteigert werden. Die Erklärung für dieses unterschiedliche Verhalten liegt darin, daß ein bestimmter Anteil des Farbstoffes im Blutplasma frei gelöst ist (Goldstein 1949) und durch die Glomerulumkapillaren *filtriert* wird. Die Hauptstückzellen dagegen scheiden den Farbstoff aktiv, d.h. unter Energieaufwendung aus. Diese Energie stammt nach den Befunden von Taggart u. Forster (1950) größtenteils aus den Prozessen der oxydativen Phosphorylierung, da nach Entkoppelung durch 2,4-Dinitrophenol die tubuläre Farbstoffausscheidung praktisch sistiert. Die Energielieferung ist offenbar nicht unbegrenzt, so daß auch die tubuläre Ausscheidung asymptotisch eine obere Grenze erreicht. Da die energetischen Prozesse an die einzelne Zelle gebunden sind, kann man noch keine Aussagen über die Ausscheidungsleistung des Hauptstückapparates in seiner Gesamtheit machen. Eine mit zunehmendem Farbstoffangebot steigende, aber schließlich begrenzte tubuläre Sekretion könnte bei konstanter Leistung der Einzelzelle auf zweierlei Weise zustande kommen:

* Mit Unterstützung durch die Deutsche Forschungsgemeinschaft.

1. Mit steigendem Farbstoffangebot ist anfänglich nur ein Teil der in der Niere vorhandenen Nephrone sekretorisch tätig, und weitere Nephrone werden laufend eingeschaltet.

2. *Alle* Hauptstücke sezernieren zwar, aber bei geringer Farbstoffkonzentration ist jedes einzelne Hauptstück nicht mit seiner ganzen Länge tätig. Bei steigender Konzentration werden ständig weitere Hauptstückabschnitte in die Sekretion einbezogen.

Nur sofern *alle* Hauptstücke auf ihrer *ganzen* Länge ausscheiden, entspräche die tubuläre Ausscheidungsrate der Sekretionsleistung der einzelnen Hauptstückzelle. In dieser Hinsicht konnte Rollhäuser (1957, 1960) an der Rattenniere ein bestimmtes Ausscheidungsmuster für Phenolrot beobachten:

1. Bei der Ratte sezernieren stets *sämtliche* Hauptstücke, unabhängig vom Farbstoffangebot.

2. Jedes einzelne Hauptstück scheidet vornehmlich in seinem gestreckten Teil im Gebiet der Markstrahlen und des Außenstreifens aus. Nur bei höherer Phenolrotdosierung werden auch die gewundenen Teile in der Rinde einbezogen. Die Anfangsabschnitte der Hauptstücke beteiligen sich nicht an der Sekretion.

3. Die Rotfärbung der Zellen in den sezernierenden Abschnitten ist bei kleinem Farbstoffangebot schwach, wird mit steigenden Dosen intensiver, doch nur bis zu einem gewissen Grad. Darüber hinaus bewirken selbst höchste Dosen keine gesteigerte Rotfärbung der Zellen.

4. Maximale Rückresorptionsleistung der Hauptstücke (Glukosebelastung) hat keinen Einfluß auf das Ausscheidungsmuster für Phenolrot.

Mit der vorliegenden Studie soll nun geklärt werden, ob sich das Ausscheidungsmuster für Phenolrot bei der Rattenniere ändert, wenn nach einseitiger Nephrektomie das verbliebene Organ die gesamte Elimination allein übernehmen muß. Weiterhin fragt sich, wie sich das Ausscheidungsbild während der folgenden kompensatorischen Hypertrophie des Restorgans darstellt. A priori bestehen nach einseitiger Nephrektomie 2 Möglichkeiten:

a) Die Ausscheidungszone in den Hauptstücken bleibt unverändert, d.h. es sezernieren vornehmlich die gestreckten Abschnitte im Markstrahl und Außenstreifen, wie es bei nicht operierten Tieren der Fall ist. Unter diesen Umständen müßte die Ausscheidungszeit für Phenolrot verlängert sein.

b) Die Niere versucht, den Farbstoff möglichst schnell zu eliminieren. Dabei müßten dann weitere Nephronabschnitte wie die proximalen gewundenen Hauptstückteile oder gar die Mittelstücke sekretorisch in Funktion treten.

Der früher erhobene Befund (Rollhäuser 1957), wonach eine maximal gesteigerte Rückresorptionsleistung die Ausscheidungsfunktion der Hauptstücke nicht beeinträchtigt, läßt vermuten, daß die Niere harnpflichtige oder körperfremde Substanzen unter allen Umständen zu sezernieren bestrebt ist, selbst wenn die Rückresorption darunter leiden müßte. Da die Rückresorption im wesentlichen in den gewundenen Hauptstückabschnitten, die Exkretion dagegen besonders in den gestreckten Abschnitten erfolgt, so wäre bei der kompensatorischen Hypertrophie zu untersuchen, ob sich die Hauptstücke in allen Abschnitten gleichmäßig vergrößern oder ob die gestreckten Abschnitte wegen der offenbar

wichtigeren Ausscheidungsfunktion bevorzugt sind. Diese Frage berührt naturgemäß auch die Phenolrotausscheidung während der Hypertrophie. Bevor wir das Sekretionsmuster für Phenolrot näher beschreiben, sollen daher einige Beobachtungen über den morphologischen Ablauf der kompensatorischen Nierenhypertrophie vorausgeschickt werden.

Material und Methode

Versuchstiere: Weibliche Ratten mit einem Gewicht von 174—242 g. Entfernung der linken Niere in Äthernarkose. Sofortige Fixierung einer Längshälfte der entfernten normalen Niere und später der hypertrophierten Niere in 4%iger Formalinlösung, in Bakerscher Lösung oder in Susa. Die andere Hälfte wurde mit dem Gefriertrocknungsverfahren fixiert. Färbung der 10 μ dicken Serienschnitte mit Hämatoxylin-Eosin unter konstanten Bedingungen. — Der Durchmesser der Nierenkörperchen und der Hauptstücke wurde mit dem Okularschraubenmikrometer an jedem 10. Serienschnitt bestimmt. Variationsstatistische Auswertung der Ergebnisse. Ein radiär durch Mark und Rinde gelegter Längsschnitt soll als Hauptschnitt bezeichnet werden. Derartige Hauptschnitte wurden mit dem Zeichenapparat nach EDINGER bei 30-facher Vergrößerung gezeichnet unter besonderer Berücksichtigung der einzelnen Zonen: Rinde, Außenstreifen und Innenstreifen des Markes. Die mittlere Breite (Dicke) dieser Zonen wurde planimetrisch bestimmt, indem der ermittelte Flächeninhalt eines Zonenrechtecks (Abb. 1) durch die stets gleiche Länge l dividiert wird. Auf diese Weise läßt sich die relative Dickenzunahme der einzelnen Zonen während der Hypertrophie errechnen. Ferner wurden auch die Gesamtflächen jeweils von Rinde und Außenstreifen auf dem Hauptschnitt planimetriert. Das ergibt für die Massenzunahme von Rinde und Außenstreifen zuverlässigere Werte, da die hypertrophierende Niere sich in allen 3 Dimensionen vergrößert und somit wenigstens zwei erfaßt werden. Um für die Volumenzunahme einen ungefähren Anhalt zu bekommen, wurde das Organ als Rotationsellipsoid aufgefaßt und das Volumen nach $V = \frac{4}{3} \pi a b^2$ errechnet, wobei die Halbachsen a und b auf dem Hauptschnitt vermessen wurden. Auf diese Weise kann z. B. das Volumen der Rinde bestimmt werden, indem vom Gesamtvolumen das Volumen von Außenstreifen + Innenstreifen + Papille subtrahiert wird. Die so erhaltenen Partialvolumina der Rinde oder des Außenstreifens stellen zwar nur grobe Näherungswerte dar, doch haben sie sich für unsere Zwecke als brauchbar erwiesen, zumal bei den meisten Nieren auch die Gewichtshypertrophie bestimmt wurde.

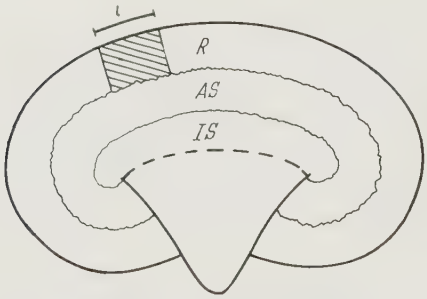

Abb. 1. Schema zur Dickenbestimmung von Rinde und Außenstreifen des Markes. Näheres im Text

Die Phenolrotausscheidung wurde nach Injektion von Phenolsulfonphthalein „Bayer" (30 mg pro kg Körpergewicht) in die Schwanzvene untersucht. In bestimmtem Zeitabstand nach Ende der Injektion, meistens nach 1 min, wurden die Tiere durch Nackenschlag getötet. Aus der Niere wurde eine zentrale, etwa 1 mm dicke Scheibe herausgeschnitten und diese in noch weitere Teilstücke zerlegt, die sofort im Gefriertrocknungsverfahren fixiert wurden. Nach beendeter Trocknung Einbettung in hartes Paraffin. 20 μ dicke Schnitte wurden trocken auf Objektträger aufgeklebt, in Xylol entparaffiniert, über Ammoniakdampf alkalisch gemacht und in Caedax, der ebenfalls über Ammoniak gehalten wurde, eingedeckt. Mit dieser Methode gelingt es, das Phenolrot in sezernierenden Zellen auf dem Schnitt karminrot darzustellen. Da der Schnitt nie mit Wasser in Berührung kommt, wird der Farbstoff nicht herausgelöst. Weitere Einzelheiten der Methodik gibt ROLLHÄUSER (1957). Mikrophotographien mit dem Spiegelreflexansatz zur Leica auf Agfa-Color-Kunstlichtfilm. Von den Farbdias wurden Vergrößerungen auf Silbereosinplatten und von diesen Schwarzweiß-Kopien angefertigt. Alle farbstoffsezernierenden Tubuli erscheinen auf der Kopie dunkel.

Befunde

1. Morphologische Veränderungen während der Hypertrophie

Die Gewichtszunahme der hypertrophierenden Nieren ist beträchtlich. Bereits nach 2 Wochen stieg das Gewicht um 50% und nach 3 Wochen um 60%. Nach Wendt (1952) beruht die Gewichtszunahme in den ersten Tagen der Hypertrophie vor allem auf einer Vermehrung des relativen Wassergehalts, die im Sinne Benninghoffs (1949) als funktionelle Schwellung des Nierengewebes aufgefaßt wird. In den späteren Stadien ist die Hypertrophie eine echte Gewebsvermehrung, wobei der Wassergehalt immer noch geringfügig erhöht bleibt. Bei der weißen Maus fand Wendt (1952) eine maximale Gewichtszunahme der Niere von 75% nach 40 Tagen. Bei nicht voll ausgewachsenen Tieren muß man beachten, daß die kompensatorische Hypertrophie mit der normalen Zunahme des Organgewichts während der Versuchszeit interferiert. Darauf hat bereits Moberg (1936) hingewiesen.

Die makroskopische Vergrößerung der Niere macht sich vor allem in der Breite und Dicke, weniger in der Länge bemerkbar. So fand Niessing (1941) bei der Maus bis zum 8. Tag der Hypertrophie eine Vergrößerung in der Länge von 11%, in der Breite von 14% und in der Dicke von 22%. Bei unseren Versuchen ergab sich eine durchschnittliche Vergrößerung in der Länge von 3% und in der Breite von 12%. Die Dickenzunahme konnte aus methodischen Gründen (s. Abschnitt Methode) nicht bestimmt werden. Das Hauptaugenmerk richteten wir weniger auf die Gesamthypertrophie als auf die Partialhypertrophie der Rinde und des Außen- und Innenstreifens des Marks. Zu dieser Untersuchung führte folgende Überlegung: Im Rindenlabyrinth liegen außer den Nierenkörperchen sämtliche gewundenen Abschnitte der Harnkanälchen, sowohl die Mittelstücke (Tubulus contortus II) als besonders die Hauptstücke (Tubulus contortus I), die massenmäßig stark überwiegen. Im Außenstreifen des Marks finden sich in erster Linie die gestreckten Teile der Hauptstücke, daneben mit geringerem Volumen gestreckte Teile der Mittelstücke und Sammelrohre. Eine Hypertrophie der Rinde käme demnach im wesentlichen durch eine Massenzunahme der gewundenen Hauptstücke zustande, eine Hypertrophie des Außenstreifens durch Vergrößerung der gestreckten Hauptstückabschnitte. Da die Hauptstücke, wie einleitend erwähnt, in ihrem Verlauf nicht überall die gleiche Funktion ausüben: gewundener Teil — Rückresorption, gestreckter Teil — Sekretion, so wäre zu vermuten, daß Rinde und Außenstreifen in *verschiedenem* Ausmaß hypertrophieren.

Tatsächlich ergaben sich bei der Vermessung auf dem Hauptschnitt für die einzelnen Zonen bemerkenswerte Unterschiede. In der folgenden Tabelle sind die durchschnittlichen Meßwerte bei einer Hypertrophiedauer von 5 und 42 Tagen aufgeführt.

Am 5. Tag nach der Nephrektomie ist die Dicke der Rinde um durchschnittlich 5% vergrößert, die des Außenstreifens dagegen um 19%. Nun besagen die Werte für die Zonendicke nicht allzuviel über das wirkliche Ausmaß der Hypertrophie. Die Rinde als oberflächlichste Zone kann bei gleichbleibender Dicke trotzdem am Volumen zugenommen haben, weil sie sich bei der hypertrophischen Niere über eine größere Oberfläche erstreckt. Je weiter papillenwärts die schalenförmigen Zonen gelegen sind, desto geringer wird die Möglichkeit, sich in der Schalen-

fläche zu entfalten, desto stärker muß die *Dicken*zunahme werden, wenn alle Zonen gleichmäßigen Volumenzuwachs besitzen sollen.

Einen besseren Anhalt für den Grad der Hypertrophie liefert die Vermessung der Gesamtfläche jeder einzelnen Zone auf dem Hauptschnitt. Auch dabei zeigt sich wieder, daß der Außenstreifen mit einer Flächenzunahme von 25% stärker hypertrophiert als die Rinde mit 11%.

Unter starker Vereinfachung können wir die einzelnen Nierenzonen als ineinandergeschichtete Rotationsellipsoide auffassen und auf diese Weise ungefähr die Volumenzunahme ermitteln. Die Berechnung ergibt auf Grund der Dimensionen des Hauptschnitts für die Rinde eine Volumenzunahme von 24%, für den Außenstreifen eine solche von 42%.

Tabelle. (Alle Zahlenangaben in Meßwerten)

Dauer der Hypertrophie	Dicke der Rindenzone		Dicke des Außenstreifens	
	normal	hypertr.	normal	hypertr.
5 Tage	4,85	5,1	2,7	3,2
42 Tage	4,9	5,0	3,0	3,25

Dauer der Hypertrophie	Fläche der Rindenzone auf dem Hauptschnitt		Fläche des Außenstreifens auf dem Hauptschnitt	
	normal	hypertr.	normal	hypertr.
5 Tage	167	186	73	91
42 Tage	170	189	78	95

Dauer der Hypertrophie	Volumen der Rinde		Volumen des Außenstreifens	
	normal	hypertr.	normal	hypertr.
5 Tage	3670	4560	1160	1650
42 Tage	3580	4680	1190	1740

Bei 42-tägiger Hypertrophie findet sich ein weiterer geringfügiger Volumenzuwachs der Rinde und des Außenstreifens. In jedem Fall ist jedoch die Hypertrophie des Außenstreifens stärker als die der Rinde. Aus diesen Befunden muß man schließen, daß die im Außenstreifen gelegenen gestreckten Hauptstückabschnitte hochgradiger hypertrophieren als die gewundenen Hauptstücke in der Rinde. (Die in beiden Zonen gelegenen Mittelstücke sind dabei vernachlässigt, da sie massenmäßig gegenüber den Hauptstücken zurücktreten.)

Aus älteren Untersuchungen geht einwandfrei hervor, daß die Volumenzunahme der Hauptstücke sowohl auf einer Verlängerung als auf einer Durchmesservergrößerung beruht. Für die Ratte gibt ARATAKI (zit. nach MOBERG 1936) eine Durchmesserzunahme von 17% an. E. PETERS (1929) findet bei der Maus nur 6%. Diese Autorin hat außerdem an Isolationspräparaten der Tubuli beobachtet, daß sich die Hauptstücke nach einseitiger Nephrektomie um 54% verlängern können (nach 2-monatiger Hypertrophie). Auch bei den vorliegenden Untersuchungen ergab sich zu verschiedenen Zeitpunkten der Hypertrophie (5, 15, 22, 62 Tage) eine signifikante Vergrößerung der Hauptstückdurchmesser um 15%. Alle früheren Befunde beziehen sich auf das Hauptstück als ganzes, eine Differenzierung zwischen gewundenem und gestrecktem Teil wurde nicht vorgenommen. Es fragt sich nun, ob die vorwiegende Hypertrophie der gestreckten Hauptstückabschnitte durch eine besondere Aktivität hervorgerufen wird. Da nach BENNINGHOFF (1949) Zellen mit gesteigerter Leistung eine funktionelle Kernschwellung aufweisen, wurden die Volumina von je 100 Kernen der gewundenen und gestreckten Hauptstücke im normalen und hypertrophischen Zustand miteinander verglichen. Dabei ergab sich, daß sich die Kernvolumina nach 22-tägiger

Hypertrophie in den gewundenen Hauptstücken um 10,5% vergrößern, in den gestreckten hingegen um 23%.

Damit erweist sich der gestreckte Teil des Hauptstücks funktionell aktiver als der gewundene. Seine Massenzunahme ist daher in den ersten Stadien der kompensatorischen Hypertrophie besonders ausgeprägt. Aber auch in späteren Stadien übertrifft er noch deutlich die gewundenen Hauptstücke. Diese Feststellungen bekräftigen die Annahme, daß die Nieren bestrebt sind, unter allen Umständen, in unserem Fall bei Mangel an harnbereitendem Gewebe, die tubuläre *Ausscheidungsrate* soweit wie möglich aufrechtzuerhalten, denn für die Ausscheidung sind in erster Linie die gestreckten Hauptstücke verantwortlich. Die kompensatorisch gesteigerte Rückresorptionsleistung im gewundenen Teil tritt demgegenüber offenbar zurück. Das will nicht besagen, daß die Gesamtrate der Rückresorption in der verbliebenen Niere zur Kompensation nicht ausreicht. Es ist durchaus denkbar, daß die Leistungsreserve für den resorptiven Stofftransport in der einzelnen Hauptstückzelle groß genug ist, um das vermehrte Angebot auch ohne Gewebshypertrophie zu bewältigen. Hierfür sprechen die Angaben der Physiologen (s. Smith 1951), wonach für jeden tubulär resorbierten Stoff wahrscheinlich ein eigenes zelluläres Transportsystem zur Verfügung steht, für die tubulär sezernierten Stoffe hingegen nur ein gemeinsames Transportsystem, um das die einzelnen Stoffe miteinander konkurrieren. Wenn dieses bei erhöhter Beanspruchung ausgelastet ist, müßte die Masse sezernierender Zellen vermehrt werden, damit die Ausscheidung mit dem Stoffangebot etwa Schritt halten kann.

Weitere morphologische Befunde bei der Hypertrophie. Die Hypertrophie des Hauptstückapparates erfolgt wenigstens teilweise durch Zellvermehrung. 5 bis 14 Tage nach Nephrektomie fanden sich zahlreiche Mitosen, bis zu 4 in einem Gesichtsfeld, vor allem in der Übergangszone vom gewundenen zum gestreckten Teil.

Die von mehreren Autoren beschriebene Durchmesserzunahme der Nierenkörperchen [Arataki (1927): 33%, Peters (1929): 25%, Niessing (1941): 18% bei der Maus] war bei unseren Versuchen nicht so ausgesprochen. Im Mittel zeigte sich eine Vergrößerung des Durchmessers um 11% (5—42 Tage nach Nephrektomie). Angesichts der erheblichen Variationsbreite, die auch v. Moellendorff (1930) angibt, konnte diese Zunahme statistisch nicht gesichert werden. Im Gegensatz zum Tubulussystem scheint eine kompensatorische Vergrößerung der Nierenkörperchen — d. h. eine Vergrößerung der Filtrationsfläche der Glomerulumgefäße — keine besondere Rolle zu spielen. Die glomeruläre Filtrationsrate kann funktionell ohne weiteres durch einen erhöhten Filtrationsdruck und eine schnellere Blutströmung gesteigert werden. Im folgenden Abschnitt wird darauf nochmals eingegangen.

Die Frage, ob sich die Nierenkörperchen während der Hypertrophie durch Spaltung vermehren, wie es Niessing (1941, 1944) bei der Maus beobachtete, kann an Hand der untersuchten Serienschnitte nicht mit Sicherheit geklärt werden. Am 5. Tag der Hypertrophie fanden sich indessen bei einem Tier zwei eng benachbarte Nierenkörperchen, die durch eine kurze Kapselbrücke hintereinandergeschaltet waren (Abb. 2). Hier könnte man an eine Spaltung denken. Auch die Nierenkörperchen auf Abb. 3 ähneln sehr den hantelförmigen Gebilden, die

NIESSING beschrieben hat. Außerdem sahen wir in einem Fall am 42. Tag Nierenkörperchen, bei denen man vermuten kann, sie hätten in ihrer Mitte an der Bowmanschen Kapsel und am Glomerulum eine Eindellung erfahren, die auf eine Durchschnürung hindeutet. Im ganzen betrachtet sprechen unsere Beobachtungen dafür, daß sich Nierenkörperchen durch Spaltung vermehren können. Doch sind diese Befunde relativ selten.

2. Die Phenolrotausscheidung während der Hypertrophie

Die Phenolrotausscheidung wurde in verschiedenen Stadien der Hypertrophie untersucht: 2 Std, 5, 10, 14, 22, 42 und 62 Tage nach einseitiger Nephrektomie. Entsprechend diesen Sta"en ist die Beschreibung der Befunde gegliedert.

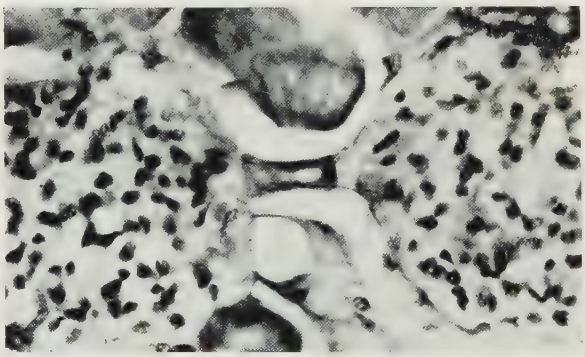

Abb. 2. Zwei durch eine Kapselbrücke verbundene Malpighische Körperchen in einer hypertrophierenden Niere. Fünfter Tag nach einseitiger Nephrektomie

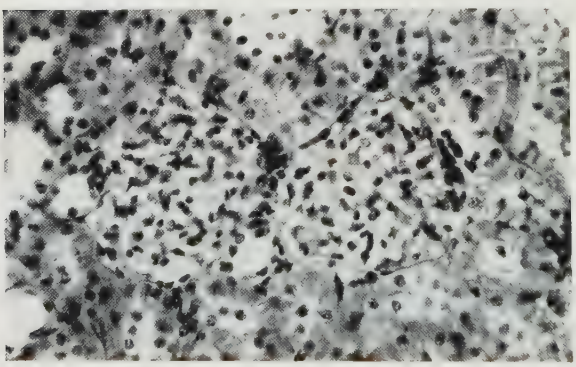

Abb. 3. Hantelförmiges Nierenkörperchenpaar. 22. Tag nach einseitiger Nephrektomie

Bereits *2 Std nach Nephrektomie* wurde Phenolrot intravenös injiziert und die Ratten genau 1 min nach Ende der Injektion getötet. Das für diesen Zeitpunkt typische Ausscheidungsbild zeigt Abb. 4. Bei der Übersichtsvergrößerung eines gefriergetrockneten Schnittes erkennt man, daß sämtliche Hauptstücke sezernieren. Das rot angefärbte Zytoplasma erscheint auf der Schwarzweiß-Reproduktion dunkel. Die kräftigste Rotfärbung findet sich in den gestreckten Teilen der Hauptstücke im Außenstreifen und in den Markstrahlen. Die intensive Färbung hört distalwärts an der Grenze zum Innenstreifen ziemlich abrupt auf. Hier befindet sich bekanntlich der Übergang der gestreckten Hauptstücke in die Überleitungsstücke (dünne Teile der Henleschen Schleife). Die gewundenen Hauptstücke in der Rinde sind ebenfalls sekretorisch tätig, aber in geringerem Ausmaß. Bei stärkerer Vergrößerung erkennt man, daß die Anfangsabschnitte des Hauptstücks, die sich unmittelbar an das Nierenkörperchen anschließen, farbstofffrei sind. Somit ergibt sich im Verlauf des Hauptstücks eine nach distal zunehmende Ausscheidung.

Der glomerulär und tubulär ausgeschiedene Farbstoff ist bereits bis in die Sammelrohre fortgeleitet, wobei er eine deutliche Konzentrierung erfahren hat.

Als Ausdruck dafür zeigen sich nach Gefriertrocknung kräftige Farbstoffniederschläge in den Sammelrohrlumina.

Dieses Ausscheidungsmuster entspricht im großen und ganzen den Verhältnissen, die Rollhäuser (1960) für die *normal* arbeitende Rattenniere 1 min nach der Phenolrotinjektion angibt. Davon abweichend findet man jedoch, daß längere Strecken der gewundenen Hauptstücke in die Sekretion einbezogen sind und daß der Farbstoff bereits nach 1 min in großer Menge bis in die Sammelrohre abgeleitet ist. Normalerweise zeigen die Sammelrohre erst 2 min nach Injektion derart kräftige Farbstoffniederschläge. Das besagt, daß nach Entfernung einer Niere der Harnfluß in den Nephronen und die Farbstoffzufuhr beschleunigt sind, die Durchblutung der verbliebenen Niere also zugenommen hat. Daraus erklärt sich auch, daß in dieser Situation praktisch das gesamte Hauptstücksystem für die Farbstoffausscheidung eingesetzt wird.

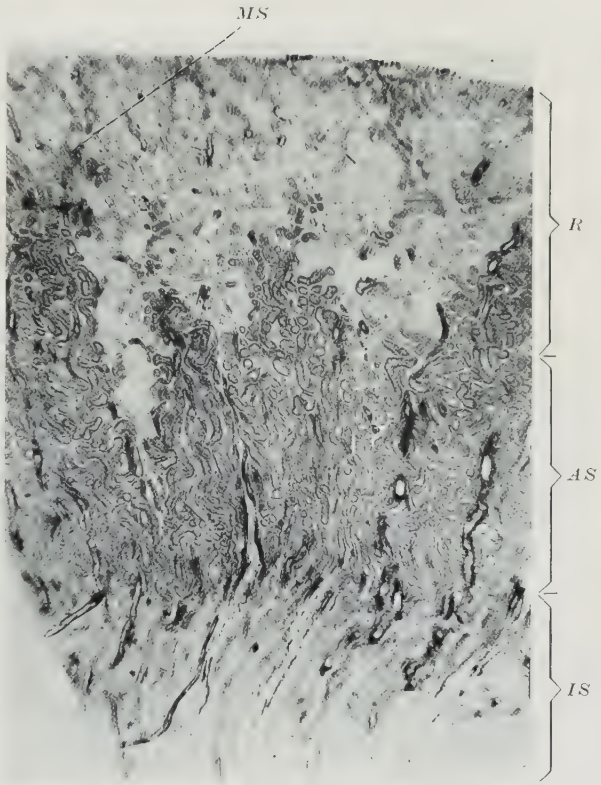

Abb. 4. Ausscheidungsbild 1 min nach Phenolrotinjektion. Zwei Stunden nach Nephrektomie. Kräftige Ausscheidung aller gestreckten Hauptstückabschnitte im Außenstreifen und in den Markstrahlen. Schwache Ausscheidung in den gewundenen Hauptstücken. Deutliche Farbstoffniederschläge in den Sammelrohren. Im Bereich des Innenstreifens zahlreiche schwächer sezernierende Mittelstücke. Vergr. 28mal

Darüber hinaus konnten wir einen Befund erheben, der bisher in der Literatur niemals erwähnt wurde. Unter bestimmten Verhältnissen sind auch die Mittelstücke im aufsteigenden Schenkel der Henleschen Schleife und im Tubulus contortus II in der Lage, Phenolrot auszuscheiden. Diese Beobachtung konnten wir nur bei Ratten machen, die kurze Zeit (etwa 2 Std) nach Nephrektomie untersucht wurden. In allen folgenden Stadien der Hypertrophie schieden die Mittelstücke nie aus. Abb. 4 zeigt im Gebiet des Innenstreifens zahlreiche gestreckte Mittelstücke, die sich durch eine mäßige Rotfärbung auszeichnen. Eine Verwechslung mit den durch massive Farbstoffniederschläge markierten Sammelrohren ist ausgeschlossen. Ganz einwandfrei erkennt man die Phenolrotausscheidung im Mittelstück auf Abb. 5. Es handelt sich hier um ein Kanälchen des aufsteigenden Schleifenteils (gestreckter Abschnitt des Mittelstücks) bei 1100facher Vergrößerung (Fluorit-Ölimmersion 114/1,32). Das Zytoplasma zeigt eine deutliche Rotfärbung im Gegensatz zu den völlig blassen dünnen Schleifenteilen, die das Mittelstück auf der Abbildung rechts und links begleiten.

Daß es sich hier um eine *Ausscheidung* handelt und keinesfalls um eine Rückdiffusion des Farbstoffs aus dem Tubuluslumen ins Zytoplasma, wird durch folgende Details gesichert: Die Phenolrotkonzentration im Lumen ist gering. Die Rotfärbung der Mittelstückzellen nimmt von der Lumenoberfläche aus in Richtung auf die Basalmembran deutlich zu. Die Basalmembran selber zeigt dort, wo sie exakt im Fokus liegt, eine tiefrote Färbung. Das alles spricht dafür, daß es sich um einen Farbstofftransport vom Ort der höchsten Konzentration an der Zellbasis in Richtung zum Lumen handelt. Ferner erkennt man bei manchen Zellen, daß der Farbstoff von der Basalmembran aus straßenförmig in das Zellinnere geleitet wird, wie es ROLLHÄUSER (1957) für das Hauptstück beschrieben hat. Als Leitbahnen fungieren nach elektronenmikroskopischen

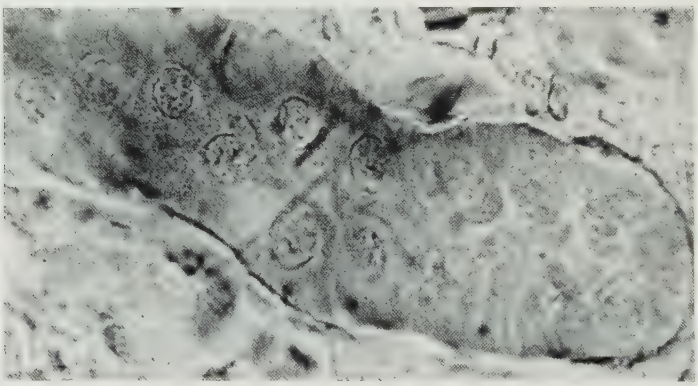

Abb. 5. Sezernierendes gestrecktes Mittelstück aus dem Außenstreifen des Markes. Zwei Stunden nach Nephrektomie. Vergr. 1100mal

Untersuchungen an der Hauptstückzelle (ROLLHÄUSER u. VOGELL 1957) die zahlreichen intraplasmatischen Doppellamellen (SJÖSTRAND u. RHODIN 1953, RHODIN 1954, BARGMANN, KNOOP u. SCHIEBLER 1955). Diese existieren auch in der Mittelstückzelle (PEASE 1955) und dürften auch hier mit dem intrazellulären Stofftransport zu tun haben.

Eine Beteiligung der Mittelstücke an der Phenolrotsekretion fand sich bisher nur unmittelbar nach einseitiger Nephrektomie. Es wäre denkbar, daß eine extrem hohe Farbstoffkonzentration im Blut dafür verantwortlich ist. Das trifft mit Sicherheit nicht zu, da die Blutkonzentration auch nach Entfernung einer Niere nicht sonderlich ansteigt und ferner selbst durch Injektion der 3fachen Phenolrotdosis (100 mg/kg Körpergewicht) beim nichtoperierten Tier keine Ausscheidung in den Mittelstücken erzielt werden kann. Steigerung der Nierendurchblutung dürfte ebenfalls als Ursache nicht in Frage kommen. Entsprechend wirksame Pharmaka induzieren keine Phenolrotsekretion im Mittelstück (ROLLHÄUSER, unveröffentlichte Befunde).

Wenn auch die zur Sekretion in den Mittelstücken führenden Faktoren einstweilen unbekannt sind, so bleibt die Feststellung, daß die Niere im Notfall außer sämtlichen Hauptstücken zusätzlich auch die Mittelstücke für die Ausscheidung mobilisieren kann.

Am 5. Tag der Hypertrophie bietet sich 1 min nach Phenolrotinjektion hinsichtlich der Hauptstücksekretion ein ähnliches Bild wie das zuvor beschriebene.

Bis auf einen kurzen Anfangsabschnitt sind alle Hauptstücke im gewundenen und besonders intensiv im gestreckten Teil an der Farbstoffausscheidung beteiligt. Die Mittelstücke weisen keine Rotfärbung auf. Die Sammelrohre enthalten kräftige Phenolrotniederschläge.

Im weiteren Verlauf bis zum 62. Tag der Hypertrophie ändert sich das Ausscheidungsmuster insofern, als die Rotfärbung der Hauptstücke unter konstanten Versuchsbedingungen sich mehr und mehr auf den gestreckten Abschnitt konzentriert und der gewundene Teil zunehmend von der Ausscheidungsfunktion befreit wird. Diesen Befund erklären wir mit der vorher erwähnten Beobachtung, daß die gestreckten Hauptstückteile in besonderem Ausmaß kompensatorisch hypertrophieren. Sie sind daher in der Lage, die Hauptmenge des angebotenen Farbstoffes zu eliminieren und die gewundenen Teile zu entlasten.

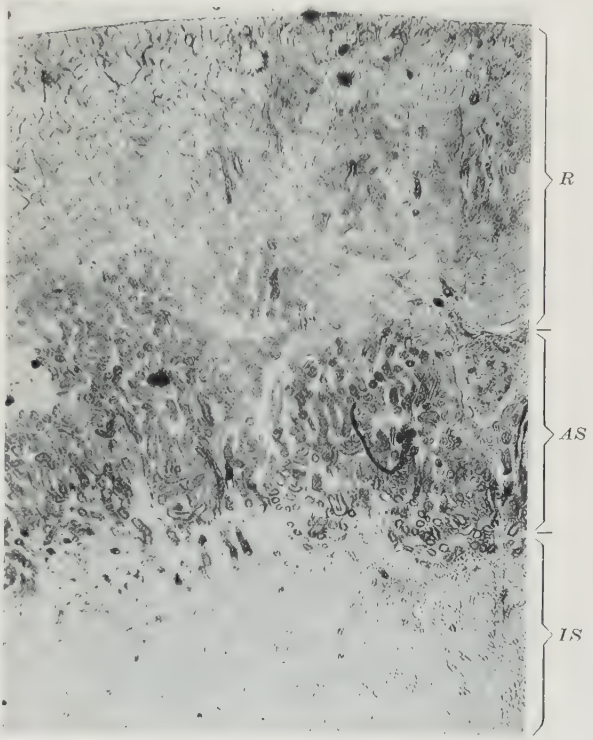

Abb. 6. Phenolrotausscheidung einer hypertrophierenden Niere (5. Tag nach einseitiger Nephrektomie). Die Farbstoffsekretion ist 15 min nach der Injektion noch nicht beendet. Rotfärbung der Zellen in den distalen Hauptstückabschnitten. Vergr. 22mal

Allerdings reicht die bevorzugte Hypertrophie der gestreckten Hauptstücke im Außenstreifen zunächst nicht aus, um den Verlust einer Niere völlig zu kompensieren. Das zeigt sich in einer verlängerten Ausscheidungszeit während der ersten Wochen der Hypertrophie. Normalerweise wird eine Phenolrotdosis von 30 mg/kg Körpergewicht innerhalb von 12 min vollständig ausgeschieden (Rollhäuser 1960). Injiziert man die gleiche Dosis am 5. Tag nach der Nephrektomie, so findet man noch nach 15 min eine kräftige Rotfärbung der Hauptstücke im Außenstreifen. In den Markstrahlen ist die Ausscheidung ganz minimal, die gewundenen Hauptstücke zeigen keine Sekretion (Abb. 6). Dieses Bild entspricht etwa den Verhältnissen, die beim Normaltier 6 min nach der Farbstoffinjektion beobachtet werden. Somit ist die Ausscheidungszeit in den ersten Tagen der Hypertrophie eindeutig verlängert. Das steht in Einklang mit den Befunden von Rowntree u. Geragthy (1912), die bei Hunden und Katzen unmittelbar nach Nephrektomie eine erniedrigte Phenolrotausscheidung beobachteten (s. auch Hinman 1926).

Der gleiche Versuch am 42. Tag nach der Nephrektomie ergibt nun, daß die Phenolrotausscheidung innerhalb von 15 min praktisch beendet ist. Die Haupt-

stücke im Außenstreifen zeigen nur noch einen Hauch von Rotfärbung, und auch in den Sammelrohren finden sich lediglich Spuren von Phenolrot (Abb. 7). In diesem Stadium der Hypertrophie hat die Masse der sezernierenden Hauptstücke, vor allem im Außenstreifen, so zugenommen, daß die Niere nunmehr in der Lage ist, den Funktionsausfall des Schwesterorgans fast vollständig auszugleichen. Bemerkenswert ist dabei, daß die hypertrophische Niere funktionell etwa dasselbe leistet, was normalerweise die Aufgabe von 2 Nieren ist, daß sie aber gewichtsmäßig beide Nieren nur zu etwa 75% ersetzt. Der Leistungszuwachs ist also größer als der Gewichtszuwachs. Das bedeutet, daß z. B. die sezernierende Zelle der hypertrophischen Niere übernormal beansprucht wird und nahe an der Grenze ihres Leistungsmaximums arbeitet. Die hypertrophische Niere besitzt eine geringere Funktionsreserve und muß daher bei übermäßigen Anforderungen relativ insuffizient werden. Auf diesen Punkt hat bereits MOBERG (1936) in ausgedehnten Untersuchungen hingewiesen.

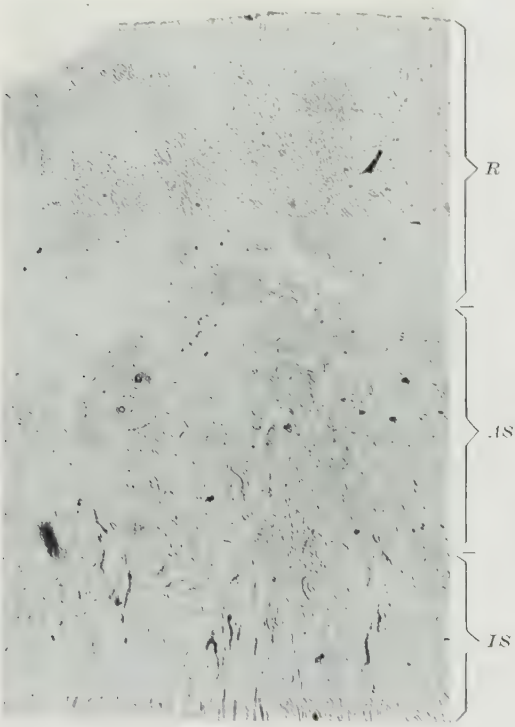

Abb. 7. Phenolrotausscheidung am 42. Tag der Hypertrophie. 15 min nach der Injektion ist die Farbstoffsekretion praktisch beendet. Vergr. 22mal

Eine weitere Versuchsreihe sollte klären, ob die hypertrophische Niere einer Schockeinwirkung in höherem Maße unterliegt als die normale. Untersuchungen von ROLLHÄUSER u. VOGELL (1960) ergaben, daß Furcht, Erregungszustände oder Schmerzen eine Einschränkung der Ausscheidungszone im gestreckten Hauptstückabschnitt hervorrufen. So zeigte sich z. B., daß bei Schmerzen, die durch mäßige Quetschung der Hinterbeine verursacht wurden, die distalen Enden der Hauptstücke keinen Farbstoff ausscheiden, wenn man die Nieren 1 min nach Injektion der Standarddosis von 30 mg/kg Körpergewicht untersucht. Normalerweise scheiden die distalen Hauptstücke bereits nach 30 sec kräftig aus. Im Schmerzzustand erscheint eine Sekretion in diesem Tubulusabschnitt frühestens 2—3 min nach der Injektion. Es ließ sich nachweisen, daß diese Ausscheidungsverzögerung die Folge einer verlangsamten Nierendurchblutung ist. Das phenolrothaltige postglomeruläre Blut gelangt verspätet an die am weitesten distal gelegenen Hauptstücke.

Die gleiche Versuchsanordnung (5 min lange Quetschung beider Hinterbeine) führte bei der hypertrophierenden Niere zu ganz anderen Ergebnissen. Untersucht wurden Ratten 5 und 14 Tage nach Entfernung einer Niere. Bereits 1 min nach der Injektion erschienen alle Hauptstücke auf ihrer *ganzen* Länge (mit der

üblichen Ausnahme des glomerulumnahen Abschnitts) rotgefärbt und sezernierten intensiv. Das Ausscheidungsbild während des Schocks war von dem einer „normal" sezernierenden hypertrophischen Niere nicht zu unterscheiden.

Wir schließen daraus, daß die hypertrophierende Niere gegen Schockeinflüsse ziemlich unempfindlich ist. Wahrscheinlich ist ihre Durchblutung so groß, daß selbst eine Minderung infolge der Extremitätenquetschung zu keiner erkennbaren Einschränkung der Phenolrotausscheidung führt. Möglicherweise sind in der Phase der Hypertrophie die nervalen Blutdruckregulatoren weniger empfindlich als im Normalzustand. Welche Faktoren im einzelnen im Spiel sind, können wir noch nicht entscheiden.

Zusammenfassung

Bei der Ratte wurde die nach einseitiger Nephrektomie einsetzende kompensatorische Hypertrophie der Niere in ihrer Auswirkung auf die einzelnen Hauptstückabschnitte bis zum 42. Tag verfolgt und das Ausscheidungsmuster nach intravenöser Phenolrotinjektion untersucht.

1. Im Verlauf der Hypertrophie nimmt der Außenstreifen des Marks stärker an Volumen zu als die Rinde. Unter Vernachlässigung der Mittelstücke bedeutet dieser Befund, daß die gestreckten Hauptstückabschnitte hochgradiger hypertrophieren als die gewundenen in der Rinde. Die Hypertrophie der Hauptstücke äußert sich in einer Kaliberzunahme und in einer Verlängerung.

2. Die Zellkerne in den gestreckten Hauptstücken zeigen nach 22tägiger Hypertrophie eine Volumenzunahme von 23%, in den gewundenen Hauptstücken eine Zunahme von 10,5%.

3. Die Durchmesser der Nierenkörperchen weisen eine so große Streuung auf, daß eine signifikante Hypertrophie nicht festzustellen ist. Anzeichen einer Spaltung wurden bei ganz vereinzelten Nierenkörperchen beobachtet.

4. Kurze Zeit nach Nephrektomie ist die Durchblutung und der Harnfluß in der verbliebenen Niere bedeutend erhöht, wie das Ausscheidungsbild nach Phenolrotinjektion ausweist.

5. Zu diesem Zeitpunkt beteiligen sich auch die Mittelstücke an der Phenolrotsekretion. In späteren Stadien der Hypertrophie konnte dieser Befund nicht mehr erhoben werden.

6. Die anfangs deutlich verlängerte Ausscheidungszeit für Phenolrot nähert sich nach 6 Wochen wieder der Norm. Damit hat die hypertrophierte Niere den Parenchymausfall nach Nephrektomie funktionell nahezu kompensiert.

7. Der Einfluß eines Schocks (Extremitätenquetschung) auf die Nierendurchblutung ist bei einseitig nephrektomierten Ratten viel weniger ausgeprägt als bei Normaltieren.

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STUDIES ON RICKETS*

I. THE FINE STRUCTURE OF UNCALCIFIED BONE MATRIX IN EXPERIMENTAL RICKETS

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With the technical assistance of ULLA F. AFZELIUS

With 12 Figures in the Text

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There is general agreement that the principal anatomical lesion in rickets is an abundance of uncalcified bone matrix. In young animals the cartilage matrix of the epiphysis as well as the bone matrix fails to calcify, and there is a concomitant disorganization of the epiphyseal apparatus during growth. Among the hypotheses which have been put forward to account for the failure of matrix to mineralize in rickets is the suggestion that an abnormal matrix is formed (ENGFELDT and ZETTERSTROM 1955). It is implied that this abnormal matrix affects the process of calcification in some undisclosed manner.

Previous studies on the fine structure of bone have shown that the inorganic crystals have an intimate relation to the periodic banding of collagen (JACKSON 1957, ROBINSON and WATSON 1955), and that during the early stages of mineralization crystals are seen within collagen fibrils (SHELDON and ROBINSON 1957). This relationship between the inorganic moiety (calcium phosphate) and the organic matrix (collagen fibrils) of normal bone has been studied in vitro by RAMACHANDRAN and SANTANAM (1956), SANTANAM (1959) and GLIMCHER, HODGE and SCHMITT (1957).

It occurred to us that rickets which had been produced experimentally would provide a situation in which the mineralization of bone matrix might be controlled and thus the relationship between the organic and inorganic components of bone might be further elucidated in vivo. The present communication reports an electron microscope study of uncalcified bone matrix in the epiphyseal plate of the tibia in young rats who have become rickety[1] on a low phosphate, vitamin D deficient diet. This study serves as a necessary precursor to investigations of the mechanism of mineralization during the healing of rickets.

Materials and Methods

Thirty weanling male rats which weighed an average of 40 grams were placed on a vitamin D deficient, low phosphate diet whose formula is shown in the Table[2]. This diet is

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[1] The confusion regarding the adjective *rickety*, as opposed to *rachitic* has arisen from the latinization of a proper Anglo-Saxon word; rickety is to be preferred both because of its priority and derivation.

[2] The authors would like to express their gratitude to Dr. CHARLES E. BILLS for generously supplying this diet of his invention. These experiments began February, 1957.

believed to be nutritionally complete except for its low phosphate content. It contains 1200 milligrams per cent calcium and 80 milligrams per cent phosphate, a ratio of 15 parts calcium to 1 part phosphate. This is approximately one-fifth the amount of phosphate normally present in a standard rickets-producing STEENBOCK-BLACK type of diet and was found to produce severe rickets more rapidly than the usual rickets-producing type of diet.

Table. *Rachitogenic Diet (F).*

Corn starch	3000 grams
Dextrose	1200 grams
Fibrin (not extracted with alcohol)	900 grams
Yeast	440 grams
Calcium carbonate	180 grams
Corn oil (100 I.U. vitamin A per cc.)	420 cc.
Salt mixture	60 grams
NaCl (iodized)	500 grams
KCl	440 grams
Ferric phosphate	20 grams
Magnesium carbonate	20 grams
Magnesium sulfate (dried)	17 grams
Cupric sulfate	3 grams

After three weeks, rats which had been fed this experimental diet and control animals were sacrificed by a blow on the head, and one-half of one tibia was examined grossly and with supra vital silver staining. All the animals on the low phosphate diet showed severe rickets when graded according to the line test.

Immediately after sacrificing the animals, the other tibia was removed and bisected. A one millimeter thick slice of the epiphyseal plate was made and small segments were removed for fixation in 1 per cent buffered isotonic osmium tetroxide, p_H 7.4, for periods up to 1 hour. The tissues were routinely dehydrated in graded alcohols and embedded in a mixture of butyl and methyl methacrylate. In order to enhance contrast in the matrix, approximately half the specimens from each animal were treated with 1 per cent phospho-12-tungstic acid in alcohol before being embedded in methacrylate. Sections were cut with a glass knife on a SJÖSTRAND microtome and observed in an RCA EMU 2—B or 3—C electron microscope. The light photomicrographs were taken by CHESTER REATHER.

Observations

The rats which were fed the low phosphate diet appeared well and continued to gain weight during the first seven days after they had been put on the diet, but thereafter did not gain weight nearly as rapidly as the control animals who ate a purina biscuit diet, however the experimental and control animals were not pair-fed. This failure to grow normally on a low phosphate diet has been reported previously (ZUCKER, HALL and YOUNG 1941). After two weeks on the experimental diet several animals showed a crusted brownish exudate about their external nares, an appearance reminiscent of the involvement of harderian glands which has been reported in pantothenic acid deficiency (MCELROY, SALMON, FIGGE and COWGILL 1941). By the twenty first day many of the animals on the low phosphate diet appeared listless, a few had diarrhea, and three animals had difficulty walking, and dragged one or both hind legs. One animal died after ten days on the diet during which time it had gained only 1.5 grams. Autopsy did not reveal the cause of death; rickets was present.

Serum phosphate determinations showed values as low as 1.1 milligrams per cent in some animals; serum calcium determinations averaged 12.5 milligrams per cent and an alkaline phosphatase determination on pooled serum was 56.9 units.

Light microscope observations

Light microscope observations on celloidin-embedded undecalcified sections of rat tibias stained with hematoxylin and eosin and v. KOSSA's silver nitrate method (for demonstrating sites of calcification) show wide borders of eosinophilic matrix about all trabeculae and a seeming disorganization of the epiphyseal

apparatus (Fig. 1) that has been described in many previous publications (Dodds and Cameron 1943, Park 1938). The cartilage columns appear to meander out of line and there is an abundance of small blood vessels surrounded by many small basophilic cells near the ends of the cartilage columns (Fig. 3). In the epiphyseal plate nearer the trabeculae of the marrow cavity there is a wide zone of eosinophilic-appearing matrix in which small basophilic cells lie, often surrounded by the eosinophilic matrix. The eosinophilic material appears identical in the light microscope to the wide osteoid borders lying about the trabeculae of the bone. The eosinophilic matrix appears different from the epiphyseal cartilage in that the cells are not surrounded by what appears as a "capsule" in the light microscope. However, at places where the recognizable epiphyseal cartilage merges with this eosinophilic matrix it is often difficult in hematoxylin and eosin sections to tell exactly where bone matrix begins and the cartilage matrix ends (Fig. 3).

From previous electron microscope studied on normal cartilage and bone (Robinson and Cameron 1956) it was apparent that there is a visible and recognizable difference in the fine structure of the two tissues, cartilage and bone. One purpose of the present study was to characterize the fine structure of the uncalcified matrix in rickets, and to determine whether this uncalcified matrix bears a resemblance to either normal cartilage or bone matrix or whether it represents another and abnormal matrix.

In polarized light the osteoid borders about the trabeculae of rickety animals appear markedly refractile and are similar in this respect to the uncalcified matrix of normal bone. Epiphyseal cartilage matrix is much more weakly refractile than bone and can be readily distinguished from bone matrix in both the normal and rickety animals by viewing the section in polarized light (Fig. 4).

Another method whereby the differences in cartilage and bone matrix can be illustrated in the light microscope is by the differing affinity of the two matrices for the dye methyl green. Cartilage matrix stains readily while the matrix of bone does not. This tinctorial difference is illustrated in Fig. 2 in a section of rickety bone where the dividing line between the cartilage and bone matrices is clearly seen.

Electron microscope observations

Specimens for electron microscopy were oriented in manner similar to that described by Robinson and Cameron (1956).

An electron micrograph of an area comparable to that seen in the light microscope in Fig. 3 (the area within the small circle) is shown in Fig. 5. With the use of phosphotungstic acid as a mordant to bring out the fine structure of the fibrils of the matrix, the usual greyish appearance of osteoblast or osteocyte cytoplasm is altered so that there is less fine detail. A complete description of the cellular components of this tissue will be deferred to the succeeding communication (Sheldon and Robinson 1961). The following electron microscope observations are limited to a description of the matrix components.

The eosinophilic-appearing matrix seen in the light microscope, which was shown to be markedly anisotropic in polarized light (Fig. 4), is composed of abundant fibrils. In Fig. 5 the small circle encloses an area which is shown at

Fig. 1

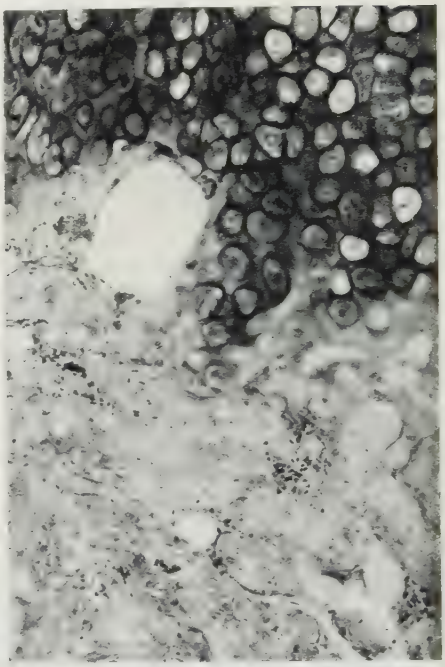


Fig.

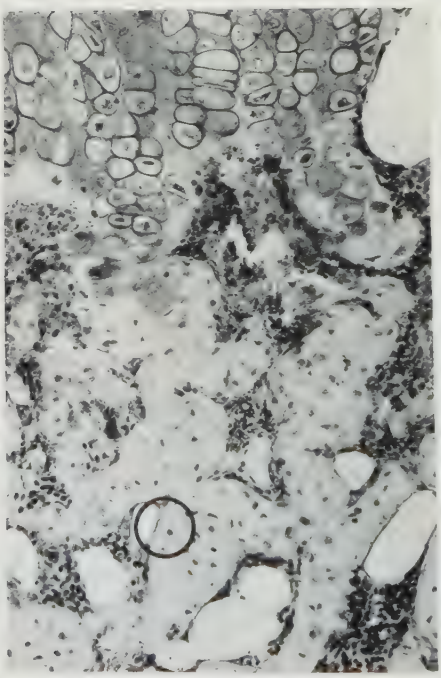
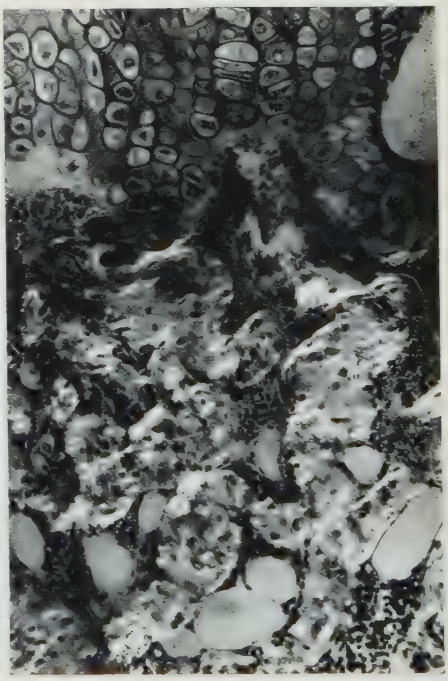


Fig. 3



Fig

Fig. 1. Light photomicrograph (× 10) of a rickety rat tibia which illustrates the markedly widened epiphyseal plate that is characteristic of rickets. Wide osteoid borders are also visible about the trabeculae. The circle encloses an area which is comparable with the area shown in succeeding photomicrographs at higher magnification

Fig. 2. Light photomicrograph (× 150) of an undecalcified celloidin-embedded section of a rickety rat

higher magnification. In addition, tangential and cross sections of these fibrils demonstrate that there is some variation in their diameter which is believed to be real and not due to the orientation of the fibrils in the section.

The difference between this matrix of fibrils which have a regular periodic structure and the matrix of cartilage is shown in Fig. 7 where one may see the more delicate fibrils of cartilage matrix which do not show the periodic banding of the larger fibrils. Robinson and Cameron (1956) remarked previously that there is no obvious line of demarcation between these two matrices in specimens from normal tissue prepared for electron microscopy in the usual manner. No limiting structure between the cartilage and rickety bone matrix was demonstrated in the present study. It is interesting to note that the fibrils of the cartilage matrix are arranged in parallel array while the fibrils of the rickety bone matrix are not in register and are arranged more haphazardly. Moreover, the fibrils of the cartilage matrix appear uniformly thin in diameter while there are thick and thin fibrils of collagen in the rickety bone matrix. Another finding of interest is that near osteoblasts, one may see an area of amorphous material wherein a few larger fibrils with a periodic structure lie among small filaments and the amorphous material. Figs. 8 and 9 show the fibrils of both types of matrix at higher magnifications. Fig. 10 shows a fibril from bone matrix in which five subbands within the major period are clearly visible in a longitudinal section, and a cross-sectioned fibril is also shown. This asymmetric subbanding within the major periodic structure of the fibril is characteristic of collagen (Bear 1952), and this characteristic has been emphasized as a "fingerprint" by which collagen may be identified morphologically. In the present study, the major repeating unit is less than 500 Å; this difference from the dimension of 640 A as reported in diffraction studies may be explained by shrinkage during preparation for microscopy.

Further micrographs which demonstrate the fine structure of the uncalcified bone matrix which is formed in rickets are shown in Figs. 11 and 12. In calcified normal bone the major bands of collagen appear in register across many collagen fibrils (Robinson and Watson 1952) but this is clearly not always the case in the matrix of rickety bone (Fig. 11), and studies on connective tissue from other sources shows that such registry is an exception rather than the rule. Karrer (1958) has reported that even an anti-parallel arrangement of the subbanding of collagen fibrils may occur in adjacent collagen fibril in connective tissue.

Differences in the diameter of the collagen fibrils are clearly demonstrated in Fig. 12, but no clear-cut relationship between the size of the fibrils and their proximity to an osteoblast has been established. On several occasions we have

tibia which has been stained with methyl green. The matrix of the cartilage appears a brilliant green while the matrix of bone (at the bottom of the picture) does not have an affinity for the stain

Fig. 3. Light photomicrograph (× 150) of an undecalcified celloidin-embedded hematoxylin and eosin stained section of rickety rat tibia treated with the v. Kossa silver stain to demonstrate calcification. Columns of cartilage cells appear at the top of the figure, abundant vascular channels which are surrounded by small basophilic cells appear in the middle, and eosinophilic uncalcified bone matrix lies at the bottom. The circle encloses an area which is comparable to the area shown in the electron micrograph Fig. 5

Fig. 4. Polarized light photomicrograph (× 150) of the same section shown in Fig. 3. This illustrates another simple method of characterizing the difference between cartilage and bone; the eosinophilic bone matrix is markedly refractile in polarized light while the cartilage matrix has only faint birefringence

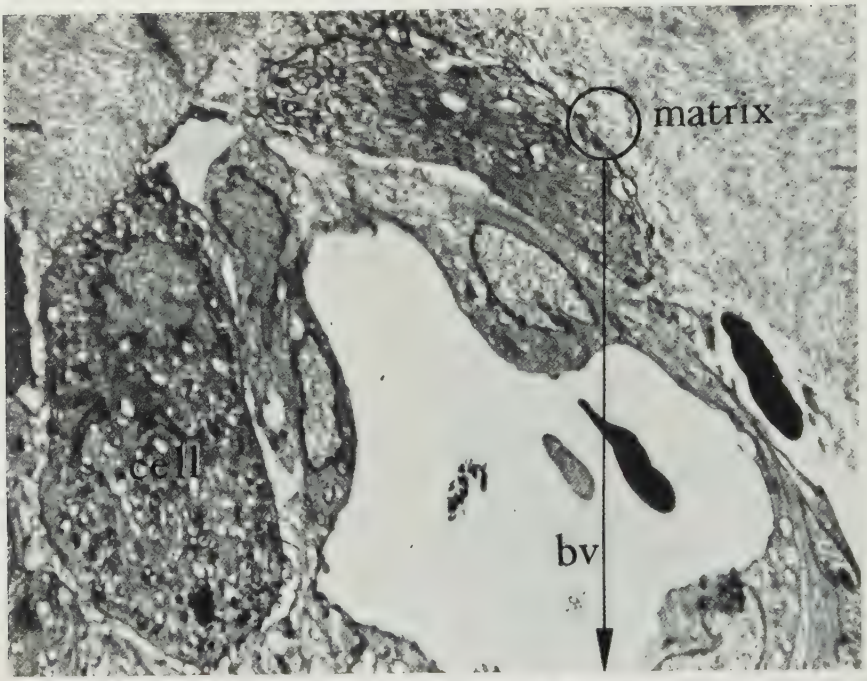


Fig. 5

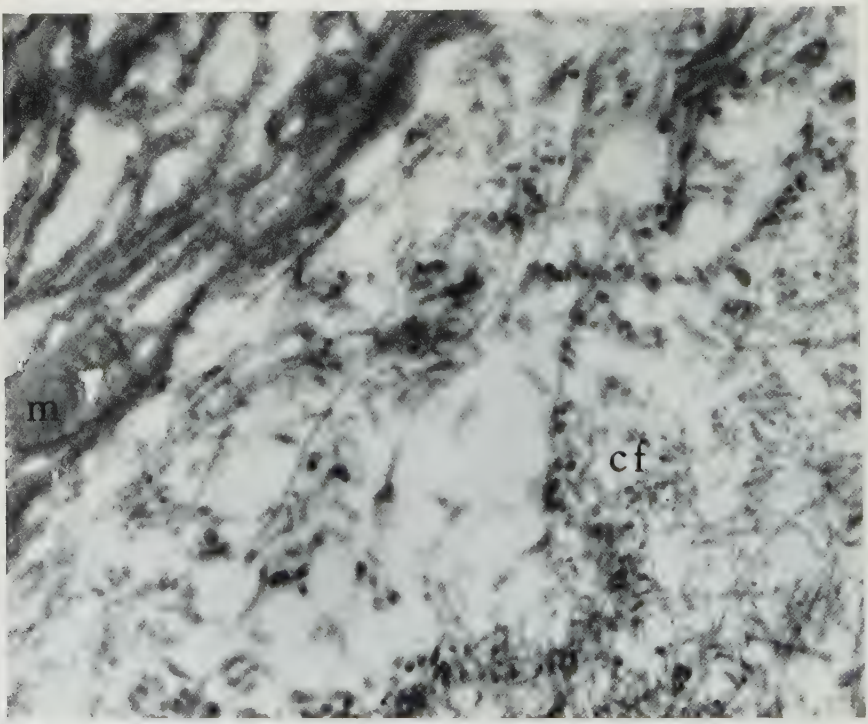


Fig. 6

Fig. 5. This is an electron micrograph (× 3,400) of a section from an area in the epiphyseal plate which is comparable to the area shown within the circle in Fig. 3. An osteoblast is shown on the left lying just outside the endothelial cell of the capillary (*bv*) which lies in the center of the Figure. A second

observed a situation quite different from that described by PORTER and PAPPAS (1959) who observed that the fibrils immediately adjacent to the fibroblast in tissue culture are small in diameter and appear larger further from the cell. Fig. 12 shows an osteocyte surrounded by fibrils seen in cross-section. The fibrils closest to the cell appear larger than those at some distance.

The electron microscope obervations presented here demonstrate that the uncalcified matrix of the epiphyseal apparatus of the rickety animal consists of two components: 1. A matrix of fibrils each of which demonstrates a major periodic structure within which as many as five subbands can be resolved. These fibrils are not arranged in an orderly manner and the population of fibrils does not appear to have a constant diameter. This matrix is identical in appearance to the osteoid borders around the trabeculae. Rickety osteoid thus appears to consist of fibrils which may be recognized as collagen by their appearance in the electron microscope but their orientation may not be so precise as in normal bone. 2. Uncalcified cartilage matrix which appears identical to the cartilage matrix of the normal epiphyseal apparatus.

Discussion

The present observations demonstrate that the rickety epiphyseal *cartilage matrix* (which would have become calcified in a normal animal) is unremarkable; the fibril size, appearance, and disposition of this rickety matrix appears identical to the matrix of normal epiphyseal cartilage matrix. The matrix of cartilage can be distinguished from bone matrix by a variety of methods; staining quality, birefringence in polarized light, and its appearance in the electron microscope. The uncalcified *bone matrix* (rickety osteoid) shows collagen fibrils as its principal component. The fibrils are recognizable as collagen because of the periodic banding within which an asymmetrically disposed subbanding appears. This appearance of collagen has been described in fibrils from normal bone (ROBINSON and WATSON 1952), rat tail tendon (SCHMITT, HALL and JAKUS 1942), human skin (GROSS and SCHMITT 1948), the lamina propria of the cornea (JAKUS 1954), the tunica propria of bronchioles (KARRER 1958), and the media of the aorta (KARRER 1958). However, the orientation of these collagen fibrils in rickety osteoid may by haphazard and unlike the alignment of collagen fibrils found in normal bone. In addition, there is a variation in the diameter of the collagen fibrils at various distances from the osteoblast, a finding which does not find a ready explanation in current hypotheses regarding fibrillogenesis (PORTER and PAPPAS 1959).

It may be pertinent to review briefly the principal advances which have led to contemporary views on the nature of bone and cartilage in general and rickets in particular. Although the clinical characteristics of rickets had been described

osteoblast lies at the upper right portion of the Figure between the capillary and the matrix and a portion of the osteoblast cytoplasm and adjacent matrix is enclosed in the small circle. This bone matrix and a portion of the adjacent osteoblast within the circle is shown at higher magnification in Fig. 6

Fig. 6. This is an electron micrograph (× 38,000) of the area enclosed within the circle of Fig. 5. A mitochondrion can be seen within the osteoblast cytoplasm at *m*. The fibrils of this matrix (*cf*) show no preferential orientation and there is some variation in their diameter. The periodic banding of these fibrils which is characteristic of native collagen can be seen in those fibrils which are sectioned longitudinally

Fig. 7

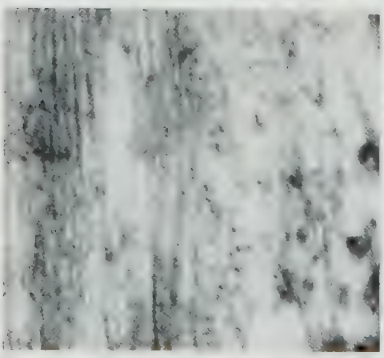


Fig. 8

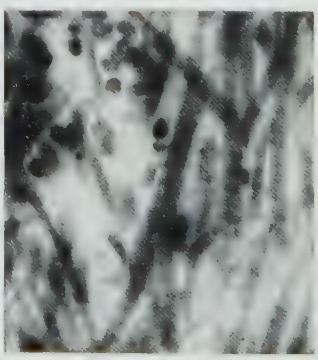


Fig. 9

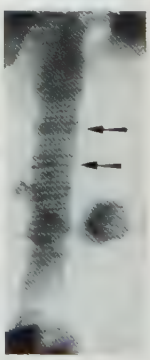


Fig. 10

Fig. 7. Electron micrograph from an area where bone and cartilage matrix meet. The cartilage matrix is visible on the left side of the figure. Although the distinction between the delicate fibrils of cartilage matrix and the coarser collagen fibrils of bone is clear, there is no membrane or other

in a classical manner by WHISTLER in his doctoral dissertation *De morbo puerile Anglorum quem patrio idiomate indigenae vocant the Rickets*[1] of 1645 and the gross pathology was well known in the 18th century, it was not until 1853 that a definitive treatise on the histologic appearance of the epiphyseal apparatus in rickets was published by VIRCHOW. As far as we can determine VIRCHOW first introduced the term osteoid in referring to the bone-like but uncalcified material seen in this tissue, but he did not define the term nor did he make any clear-cut distinction between the tissues, cartilage and bone. This is not surprising because as late as 1847 in KOLLIKER'S *Manual of Human Histology*, there is obvious confusion regarding the differences and similarities in bone and cartilage. POMMER (1885) and SCHMORL (1909) in their treatises on rickets continue to use the term osteoid but still fail to make any distinction between cartilage and bone, and it is not until one reads E. A. PARK'S Harvey Lecture (1938) that one feels that the distinction between lesions in cartilage and bone in rickets has been clearly recognized.

The understanding of the pathogenesis of rickets and the role played by calcium, phosphorus and vitamin D, have all been elucidated since 1920. In a well-documented experimental study which was carried out during the period of MELLANBY'S first successful efforts to produce experimental rickets (1921), KORENCHEVSKY (1922) commented "there is no doubt that the key to the problem of rickets is to be found in the discovery of the cause which prevents the deposition of calcium salts in the osteoid"... "The varying amount of osteoid[2] in different bones depends on the varying degree of growth of bones, differences in mechanical effects of weight, pressure and stress on bone and the processes of reconstruction of the skeleton".

This key was found with HOWLAND and KRAMER'S (1921) observations on the role played by the ions calcium and phosphorus. It is particularly relevant to note that they observed that "in rickets we believe that there is constantly a marked and for the causation of the pathologic leasion, an important deficiency in inorganic phosphorus. To this deficiency is to be ascribed the failure of calcium deposition".

[1] FRANCIS GLISSON did not publish his better known treatise *De Rachitide* until 1650.

[2] We would like to suggest that the term osteoid should be used to refer to uncalcified bone matrix (collagen) as characterized in this report, and the appropriate adjective, rickety or physiological, be always appended. Thus the thin border of uncalcified bone matrix about the trabeculae of bone in a young growing animal which is mineralizing in a normal manner is *physiological osteoid* while the wide borders of uncalcified bone matrix about the trabeculae of an animal who is not depositing mineral in a normal manner should be called *rickety osteoid*. This term does not include the uncalcified cartilage matrix which may be abundant in rickets.

structure which divides them. In the lower right corner a portion of an osteoblast (cell) may be seen. In the immediate vicinity of this osteoblast there is amorphous matrix material (arrow) in which a few filaments may be discerned (× 18,500)

Fig. 8. Higher magnification of a portion of the cartilage matrix shown in Fig. 7 which illustrates the difference between the fibril diameter of the cartilage matrix and the appearance of the fibrils of bone matrix shown in Fig. 9 (× 46,200)

Fig. 9. Higher magnification of a portion of the bone matrix shown in Fig. 7 which should be compared with the cartilage matrix at the same magnification (× 46,200)

Fig. 10. Electron micrograph of portions of two collagen fibrils from rickety osteoid showing one fibril in cross section and one in longitudinal section. Five subbands can be seen within the major period (arrows) of the longitudinally sectioned fibril. The collagen fibrils in cross section lies below the arrow (× 146,000)

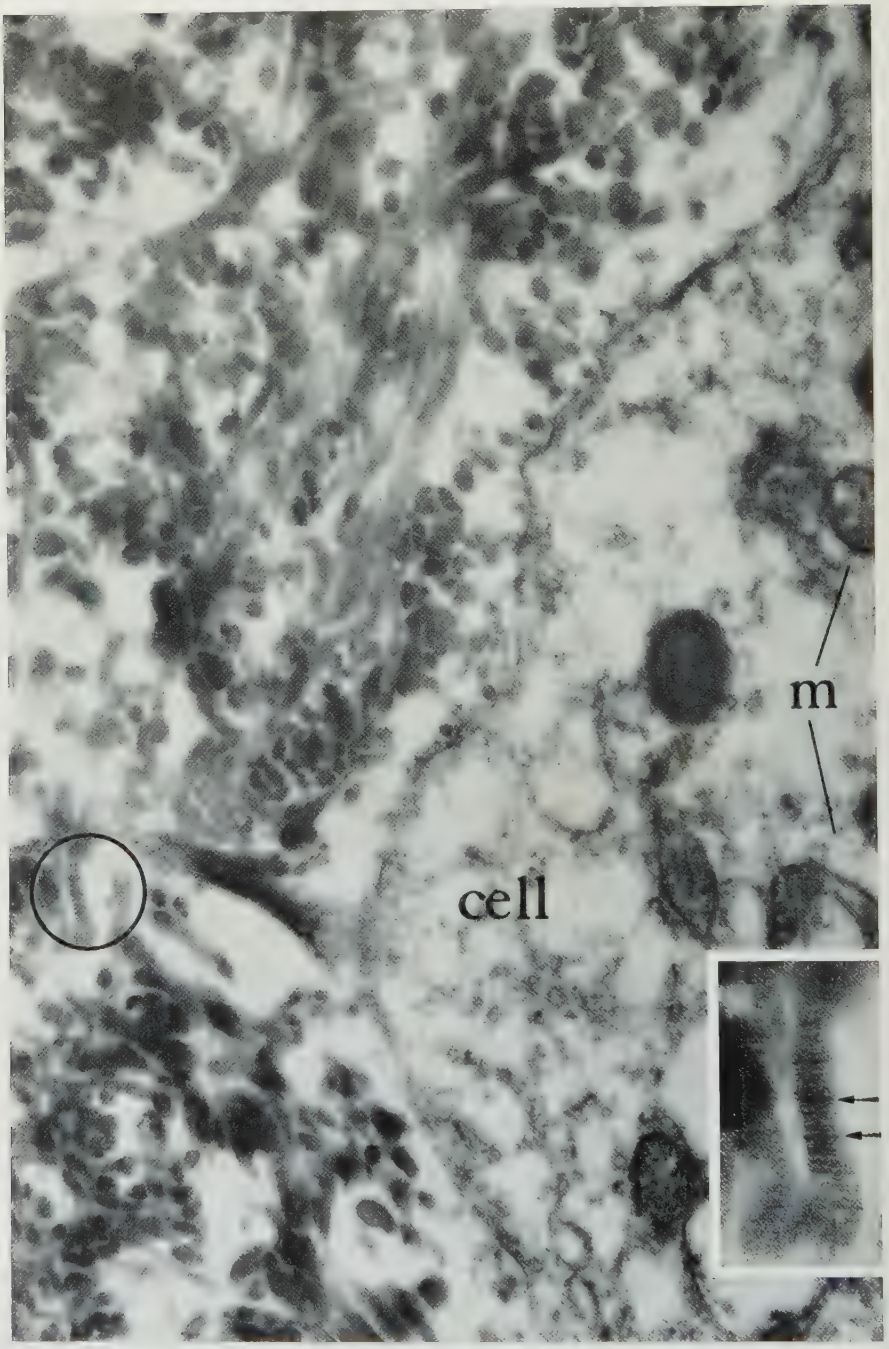


Fig. 11. Electron micrograph (× 52,500) of a portion of an osteocyte from a rickety animal in which mitochondria (*m*) can be seen. The cytoplasm of this cell which is surrounded by fibrils does not show the abundant lamellar arrangement of the granular component of the endoplasmic reticulum that is seen in many osteoblasts in normal bone. The collagen fibrils of the matrix are disposed at random outside the cell. The periodic structure of the fibrils with the characteristic subbanding can be seen in many areas. A higher magnification of a portion of the collagen fibril in the circle is shown in the inset (× 150,000) and the major period is indicated by the arrows

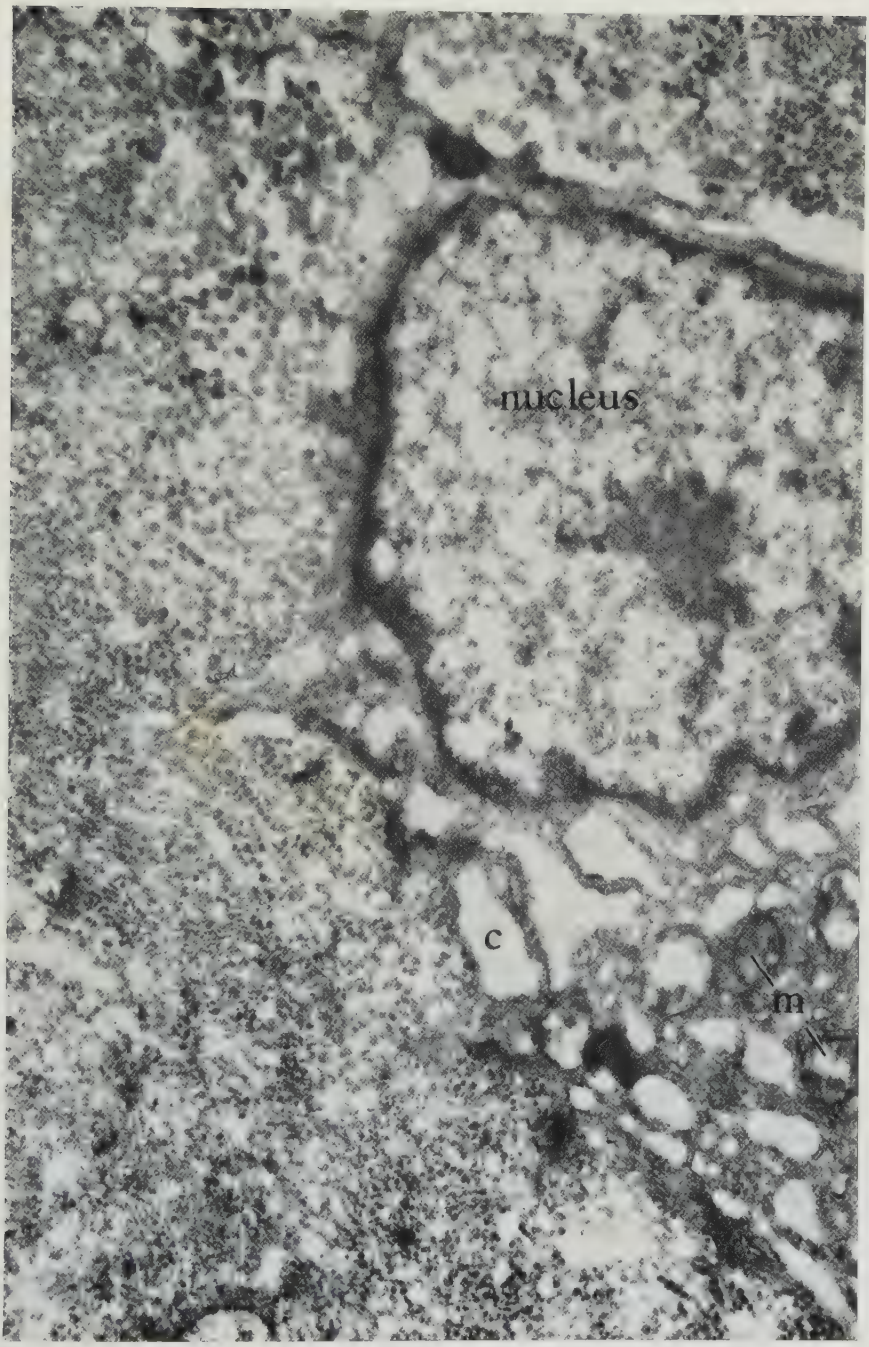


Fig. 12. Electron micrograph (× 23,000) of a portion of an osteocyte which is surrounded by collagen fibrils, the majority of which are seen in cross section. The fibrils which lie closest to the cell appear larger in diameter than those further away. Since the population of diameters in any small area appears uniform one cannot explain this appearance on the assumption that there is tapering of fibrils. Within the osteocyte cytoplasm mitochondria (*m*) and cisternae (*c*) of the endoplasmic reticulum can be seen, in addition to the centrally located nucleus

PAPPENHEIMER (1922) showed that low phosphate rickets could be healed by the administration of phosphate alone, and DODDS and CAMERON (1934) in their studied on rickets point out that there may be a reduction of growth rate as well as a failure to calcify bone in low phosphate rickets, a finding which had been demonstrated by ZUCKER, HALL and YOUNG (1941).

If we look for a definition of rickets we find a variety of descriptive definitions and several, such as the following, which make an effort to relate the pathogenesis to the anatomical lesion.

PARK (1938) defined rickets as "a disturbance in the metabolism of the growing organism of such nature that the salt equilibrium, in particular as regards the $Ca \times P$ in the circulating fluid is disturbed, and lime salts are no longer deposited in the bones".

More recently LINDQVIST (1957) has defined rickets as "a condition of vitamin D deficiency recognized by a decrease in inorganic phosphorus in serum, and certain skeletal changes which consist mainly in the continued formation of matrix with the failure to calcify it".

It does not seem necessary here to discuss the possible roles of vitamin D in phosphate and calcium metabolism but should suffice to remark that there seems to be substantial evidence that the effect of vitamin D is not directly on the organic matrix of bone but rather on the absorption and distribution of calcium and phosphate.

In returning to the problem of the direct relationship between the collagen fibrils of the bone matrix and the calcium-phosphate levels of the serum and extracellular fluids, it seems clear from previous studies on rickets that the low phosphate level in the serum alone may explain the failure of calcification. But recent studies, such as those of GLIMCHER, HODGE and SCHMITT (1957), implicate the collagen fibril as a nucleation site for the precipitation of the calcium-phosphate complex. The electron microscope observations presented here demonstrate that collagen fibrils of normal appearance are present in the uncalcified bone matrix of rickets but that one apparent difference between this matrix and normal bone matrix is a slight disarray of the collagen fibrils. The additional finding that there is a variation in fibril diameter suggests that there could be an abnormality in the rate of formation of bone matrix as well as its subsequent calcification. The lesion produced in these experiments is due to a severe dietary deficiency of phosphate (viz. serum phosphate determinations of as low as 1.1 milligrams per cent compared to a normal phosphate in a rat of 8—9 milligrams per cent). We wonder whether the fibril formation of collagen under normal conditions may be dependant on the ubiquitous phosphate energy-producing systems.

Summary

Observations with the light and electron microscope on the epiphyseal plate of rickety rats demonstrate several differences between the uncalcified cartilage and bone matrix. Uncalcified *cartilage matrix* is less refractile than bone matrix when it is viewed in polarized light. Electron microscopy shows that the fibrils of epiphyseal cartilage matrix are delicate and do not reveal the regular asymmetrical periodic structure that is so characteristic of collagen. The uncalcified *bone matrix* consists of fibrils with the regular fine structure of collagen. No

unusual features could be found in the periodic banding of the collagen fibrils of the rickety osteoid, but some variations in their diameter and array were observed.

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STUDIES ON RICKETS

II. THE FINE STRUCTURE OF THE CELLULAR COMPONENTS OF BONE IN EXPERIMENTAL RICKETS*

By

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With the technical assistance of ULLA F. AFZELIUS

With 12 Figures in the Text

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The purpose of this communication is to describe the fine structure of the cells which are associated with the collagen fibrils of rickety osteoid, to describe the topographic relationship between these cells and the extracellular matrix, and to propose a mechanism which may explain the origin of the extracellular matrix.

Materials and Methods

The materials and methods in this study have been described in the preceding communication (SHELDON and ROBINSON 1961). The only difference in the preparation of this tissue for examination in the electron microscope is that phosphotungstic acid was not used as a counter stain and therefore the details of the structure of the matrix are not so striking.

Observations

Light microscope observations on celloidin-embedded undecalcified sections of rat tibia stained with hematoxylin-eosin and VON KOSSA's silver nitrate method show abundant eosinophilic matrix material (rickety osteoid) in the epiphyseal plate and about the delicate trabeculae. The rickety osteoid merges with uncalcified matrix of the irregular cartilage columns. There are tufts of capillaries in the region of the interface between these two kinds of matrix: the uncalcified bone matrix, which is distinctly eosinophilic, and the uncalcified cartilage matrix which is faintly basophilic. Other differences in the appearance of these matrices have been described in an earlier communication (SHELDON and ROBINSON, 1961). About the perimeter of the capillary loops is a group of cells which are more basophilic and smaller than cartilage cells. These cells occasionally lie within cartilage lacunae separated by several microns from the vascular loop. There often is eosinophilic matrix material in the immediate proximity of these cells which are the osteoblasts of rickety bone. The three populations of cells: the large cartilage cells which do not appear to differ from cartilage cells in normally calcifying bone; the endothelial cells of the capillary loops, with red and white blood cells in the capillary lumina; and the osteoblasts of rickety bone, are each distinguishable in the light microscope in most instances by their appearance and by their position in relation to the other elements of the epiphyseal plate.

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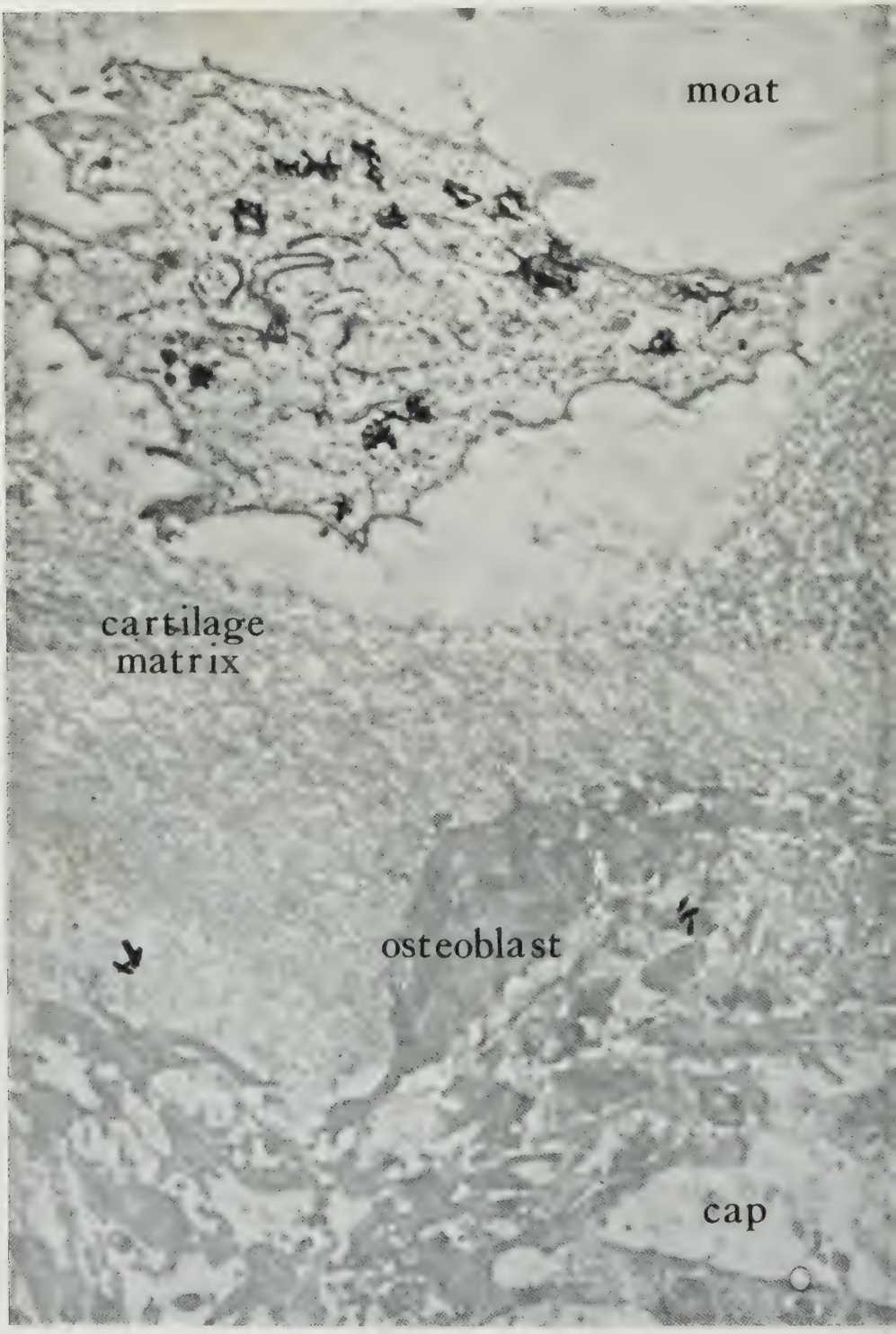

Fig. 1

In the present study we are concerned principally with the fine structure of the osteoblast in the rickety epiphyseal apparatus. The question from what cell is an osteoblast derived, and when does it become recognizable as an osteoblast, are beyond the scope of the present study; the present observations are concerned with some features of the fine structure of osteoblast cytoplasm in different stages of function and maturity.

Since new bone formation in the epiphyseal plate coincides with the invasion of the cartilage columns by capillary loops, it has been suggested that the osteoblasts may be derived from capillary endothelial cells or from leukocytes which accompany the vascular invasion or from perivascular or vascular adventitial cells. In a survey electron micrograph (Fig. 1) the vascular elements lie at the bottom of the picture and a cartilage cell within its cartilage matrix and moat lies at the top. A slightly elongated cell with abundant cytoplasmic membranes or "endoplasmic reticulum" (Porter 1956) lies at the periphery of the vascular channels; this cell is believed to be an osteoblast. Fig. 2 shows in another section another cell which lies just outside the capillary loop. This cell is large and round, contains many mitochondria, and abundant elements of the rough-surfaced component of the endoplasmic reticulum (Palade 1956). This cytoplasmic component, which has been also called the granular reticulum (Palay and Palade 1955) to distinguish it from the principal component of the golgi complex (or agranular reticulum), has been shown to consist of ribonucleoprotein (Siekevitz and Palade 1958, 1959), and to be responsible for the intense basophilia of cell types which are associated with the secretion of protein [exocrine cells of the pancreas (Palade 1956), parotid acinar cells (Porter 1956) and plasma cells (Palade 1956)]. The osteoblast differs in this respect from endothelial cells and from the "mature" cartilage cells, such as the one depicted in Fig. 1 which contain relatively little of the rough-surfaced component of the endoplasmic reticulum. These findings suggest that a cell such as the one in Fig. 2 represents an osteoblast which is not recognizable by its proximity to bone matrix but is recognizable because its cytoplasm contains an abundance of those elements which have been described in normal osteoblasts (Knese and Knoop 1958, Scott and Pease 1956, Sheldon and Robinson 1957).

There are no visible dilatations in the "cisternae"[1] of the endoplasmic reticulum of the large round cell in Fig. 2. In Fig. 3 several cells are seen which lie at some distance from the capillary loop but still do not border on the new bone matrix. These cells show what appears to be less compact cytoplasm because there are dilatations of the cisternae of the endoplasmic reticulum. Because most osteoblasts which border on new matrix show dilatations in the cisternae

[1] The term "cisternae" was introduced by Palade and Porter (1954) to designate a reservoir-like formation in a system of communicating cavities or vessels and refers to the space enclosed by the smooth inner surface of the rough-surfaced component of the endoplasmic reticulum.

Fig. 1. This survey electron micrograph shows the juncture between the cartilage columns and the capillary tufts of the epiphyseal plate in a rickety rat. A cartilage cell which contains several lipid droplets and a few elements of the endoplasmic reticulum lies within a relatively clear space (*moat*). The fibrillar cartilage matrix does not begin at the irregular cell periphery. Many portions of perivascular cells are seen near the single small osteoblast. The osteoblast shown here at very low magnification has abundant cytoplasmic membranes which have been designated the rough-surfaced component of the endoplasmic reticulum. The capillary lumen lies at the lower right corner (*cap*)

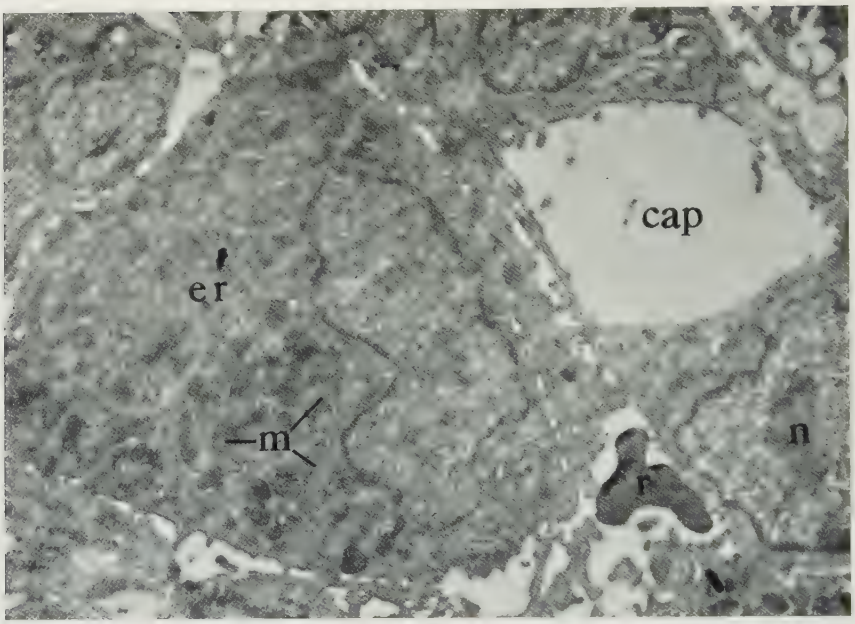

Fig. 2

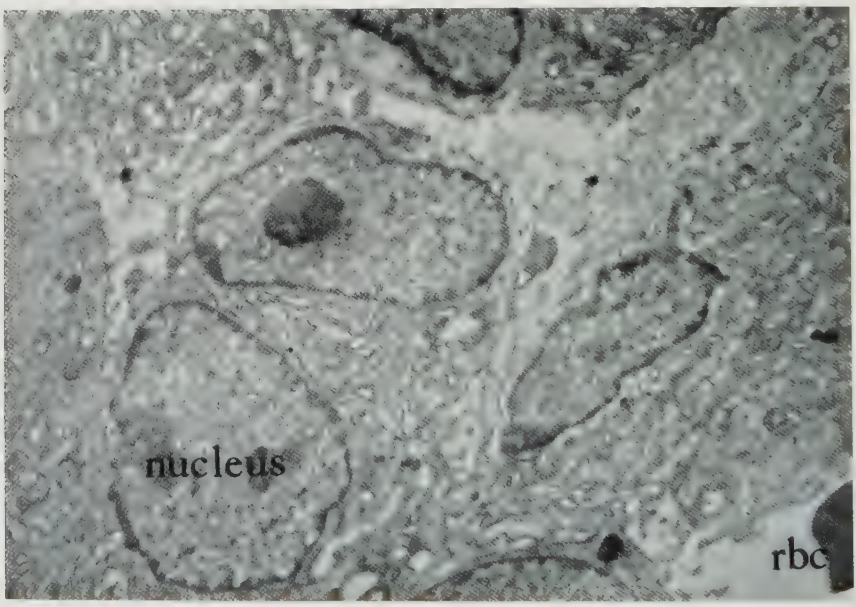

Fig. 3

Fig. 2. This electron micrograph from the epiphyseal plate of a rickety rat shows a capillary in cross section. The nucleus of an endothelial cell lies in the right lower corner (*n*), and a red blood cell (*r*) lies outside the capillary lumen. A large round cell lies just outside the capillary on the left side. This cell contains many mitochondria (*m*) and abundant elements of the rough-surfaced component of the endoplasmic reticulum (*er*), as well as many small unattached particles. The cisternae of the endoplasmic reticulum are not dilated in this cell and do not contain any amorphous material (×6400)

Fig. 3. This electron micrograph shows portions of several cells which lie in the immediate vicinity of the vascular tufts in the epiphyseal plate of a rickety rat but do not border on the bone or cartilage matrix. Several of these cells show dilatations in the space between the smooth inner surfaces of the rough-surfaced component of the endoplasmic reticulum (the cisternae). This can be seen more clearly in the next plate (×6600)

of the endoplasmic reticulum (Knese and Knoop 1958, Sheldon and Robinson 1957), we would like to suggest that dilatations in the cisternae may represent the cytoplasm in a more active secretory stage in comparison with the appearance of the cytoplasm in a "resting" osteoblast. Knese and Knoop (1958) have observed that there is very little of the rough-surfaced component of the endoplasmic reticulum present in osteocytes compared to osteoblasts.

A higher magnification of portions of rickety osteoblasts is shown in Fig. 4, where one may see nuclear chromatin, nuclear membrane pores like those described in many cell types (Watson 1955, 1959), mitochondria with their characteristic cristae (Palade 1952), and abundant elements of the endoplasmic reticulum. In these osteoblasts from a rickety animal we have the impression that the granules of the rough-surfaced component of the endoplasmic reticulum [alpha cytomembranes (Sjöstrand 1956), or ergastoplasmic membranes (Bernhard and Rouillier 1956)][1], are slightly smaller than the granules of the rough-surfaced membranes in an osteoblast from a normal animal, but differences in section thickness and other technical factors make such an impression difficult to substantiate.

Osteoblasts may be seen lying at some distance from the capillary loops, where they appear to invade the cartilage cell moat or lacuna (Fig. 5). The existence of cartilage lacunae (or moats) as real spaces has been questioned as a shrinkage artefact or as an illusion due to the different densities of matrix at different distances from the cartilage cell (Sheldon and Robinson 1958). In cartilage of the epiphyseal plate there appears to be very little matrix material visible in the immediate vicinity of these "mature" cartilage cells (Scott and Pease 1956). One wonders whether material containing very little protein lies about these cells in life, and is washed out during preparation for sectioning. In any case, osteoblasts may move into this area as seen in Fig. 5. These osteoblasts demonstrate abundant cytoplasmic granules and rough-surfaced membranous components of the endoplasmic reticulum with some dilatations of the cisternae. The cisternal contents are amorphous and moderately dense; no fine fibrillar structure has been seen within the cisternae in these osmium tetroxide-fixed specimens which were not counter-stained with phosphotungstic acid. No intracisternal granules such as those reported by Palade (1956) have been seen and no specific lesions of mitochondria such as those seen in Vitamin A deficiency (Sheldon and Zetterqvist 1956) are present in these cells from the phosphate-depleted animals.

In many osteoblasts there is a moderate dilatation of membranes of the rough-surfaced component of the endoplasmic reticulum. Many sections of rickety osteoblasts show amorphous material in these cisternae, and one may see a lamellar disposition of the endoplasmic reticulum (Fig. 7) similar to that described in pancreatic acinar cells (Sjöstrand and Hanzon 1954), parotid gland cells (Porter 1956) or plasma cells (Palade 1956). In other sections a less regular pattern without the lamellar array of the membranes is apparent (Fig. 8). Without the addition of phosphotungstic acid to enhance the contrast of the matrix it may be difficult to distinguish between the cartilage and bone matrix as illustrated in Fig. 6. Here the two types of matrix can be differentiated but not nearly so

[1] These three terms are synonymous.

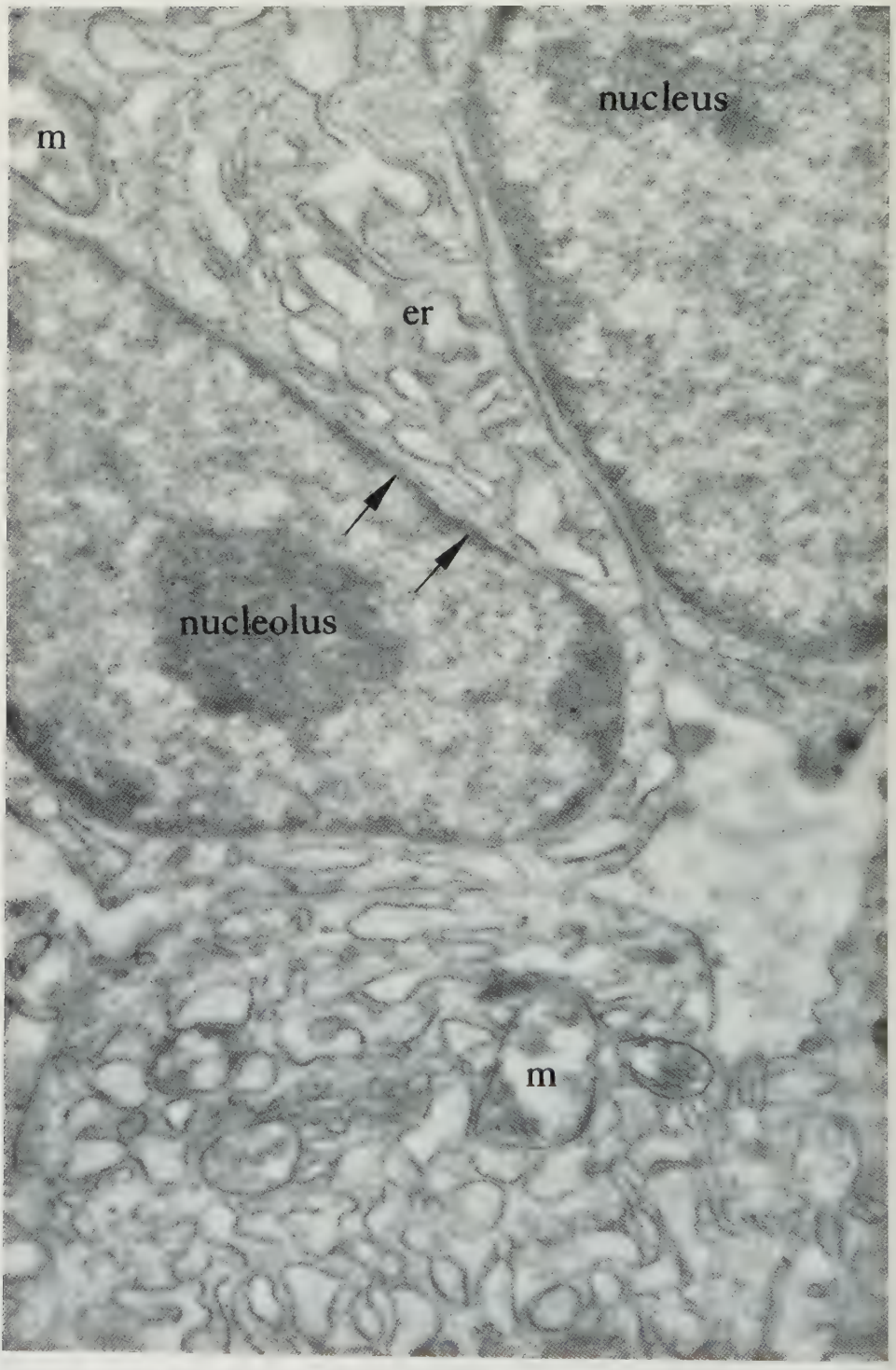

Fig. 4

clearly as when phosphotungstic acid has been used (Sheldon and Robinson 1961). In addition to the fibrillar matrix material in this electron micrograph there is amorphous extracellular material which sometimes can be seen lying between the osteoblasts and the collagenous bone matrix (Fig. 6). This suggests that there may be another constituent of the matrix; a matrix substance which does not appear fibrillar. The density of this material and its resemblance to the afibrillar material contained within the cisternae suggest that these two substances may be related. We have the impression that the dilated cisternae are seen more commonly near the amorphous component of the bone matrix.

During the course of these studies we have searched for morphologic evidence to suggest a mechanism whereby matrix material or matrix precursor may be elaborated and secreted by the osteoblast. For example, in all fields where osteoblasts lie adjacent to matrix we have searched to see whether collagen fibrils are visible within the osteoblast cytoplasm. We found no examples which could not be interpreted as an artefact of sectioning; a tangential section through a cell may give the impression that collagen fibrils are being extruded from the cytoplasm.

An alternative explanation for the mechanism whereby matrix can be produced by cells is that the matrix is secreted by the cell in a form which is not identifiable as collagen because it does not have the banding that is characteristic of collagen. Such a precursor to the collagen fibril might appear in electron micrographs of sectioned material as amorphous moderately dense material such as that seen near the cell in Fig. 6. In life this material presumably soon polymerizes to form the collagen fibrils of bone matrix, which are then recognizable by their structure.

We have seen enough of such an hypothetical material in the present study and the study reported previously (Sheldon and Robinson 1961) to demonstrate that such an afibrillar material is present. There may be a ready explanation for why more material is not visible in sections from the rickety rats which are growing very slowly. Presumably they may be synthesizing proteins at low rates. Because of this, rickety osteoblasts are not ideally suited to demonstrate morphologic evidence of secretion.

However, we have seen in a few places at the perimeter of osteoblasts an appearance of the cytoplasm which suggests that the cisternae of the endoplasmic reticulum may occasionally open up and their contents may lie in continuity with the extracellular space (Fig. 9 and 10). In Fig. 9 the material which lies outside the osteoblast is amorphous and in Fig. 10 the matrix material in the bay can be recognized as collagen.

These observations on rickets lead us to propose that previous observations on other connective tissues (Jackson 1958, S. F. Jackson 1956, 1957, Smith and Jackson 1957) and the observations presented here can best be understood in terms of the following hypothesis: collagen is secreted as an afibrillar material

Fig. 4. This electron micrograph shows a higher magnification of a portion of Fig. 3. In these cells, which are believed to be osteoblasts, one sees abundant chromatin in the nucleus and centrally located nucleoli which appear in electron micrograph simply as a more central aggregate of the same dense granular material that is seen at the border of the nucleus. Well defined nuclear pores are clearly visible (arrows). The granular membranes of the endoplasmic reticulum are somewhat dilated but no fine structure is seen in the moderately dense material within these cisternae. Mitochondria are seen at *m* (× 25000)

Fig. 5. This electron micrograph shows portions of three osteoblasts which lie at some distance from the nearest capillary tuft which if it were shown would lie below the lower right corner. In the middle of the ring of osteoblasts lie the remnants of a cartilage cell. No cell surface membrane of this cartilage cell is recognizable, and only a few elements of the endoplasmic reticulum are present. There is very little cartilage matrix material present in the immediate vicinity of the cartilage cell remnants but the delicate fibrils of cartilage matrix are visible both at the left and right perimeters of the micrograph. One might note the abundant rough-surfaced components of the endoplasmic reticulum in the three osteoblasts ($\times 7000$)

which then polymerizes in the extracellular space and becomes identifiable because of its characteristic structure. One may regard this process as similar to the conversion of fibrinogen to fibrin (HALL and SLAYTER 1959) though in the

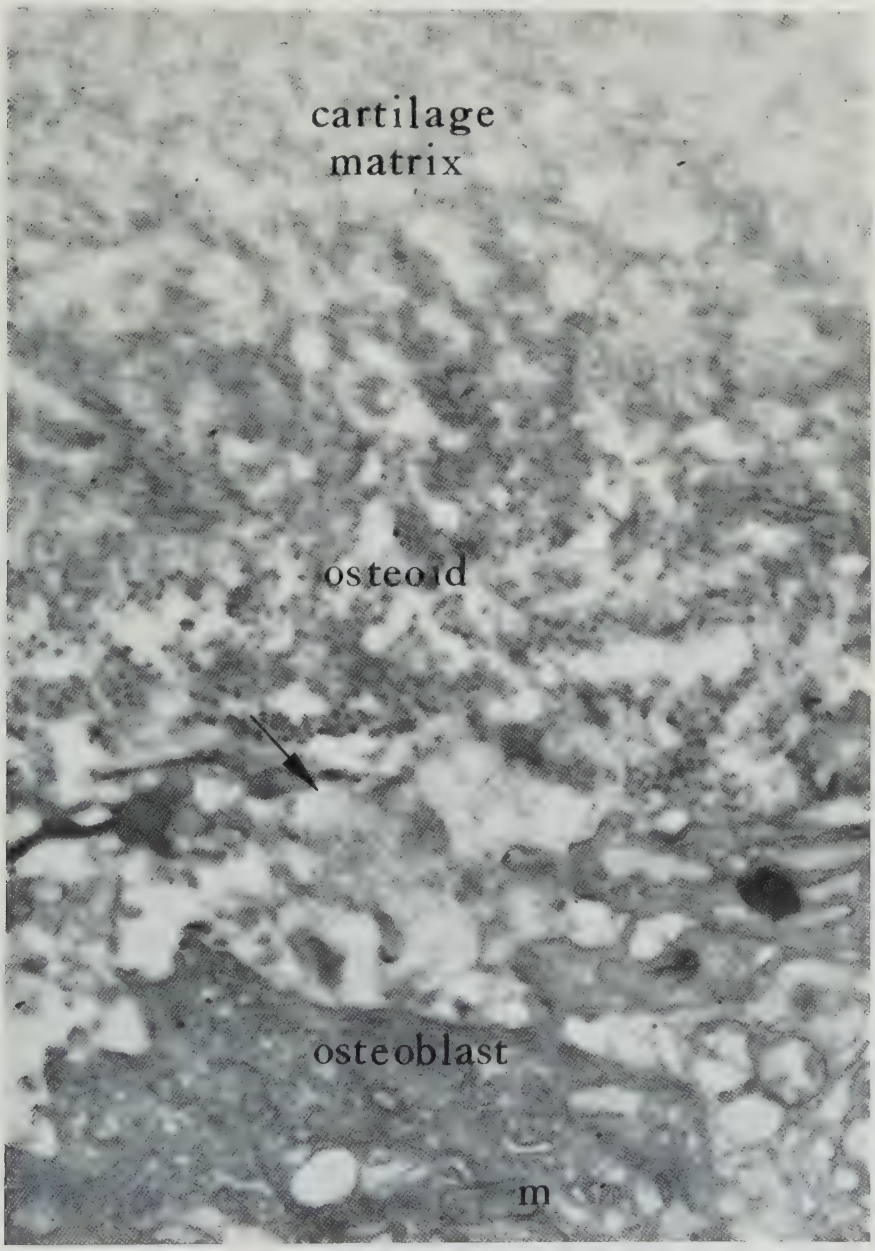

Fig. 6. This electron micrograph shows portions of two osteoblasts at the bottom of the figure. A mitochondria lies at *m*. There is some amorphous material which lies in the immediate vicinity of these cells (arrow). Above the osteoblasts in the middle of the figure there is uncalcified bone matrix (rickety osteoid) in which the banded collagen fibrils can be recognized although their banding is not so striking as in those specimens which have been treated with phosphotungstic acid. Uncalcified cartilage matrix appears at the top of the figure ($\times 18000$)

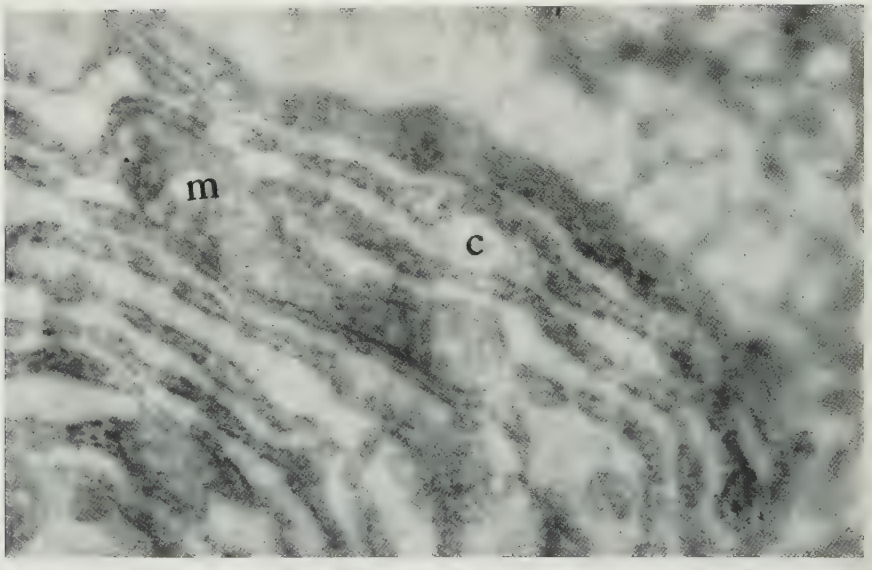

Fig. 7

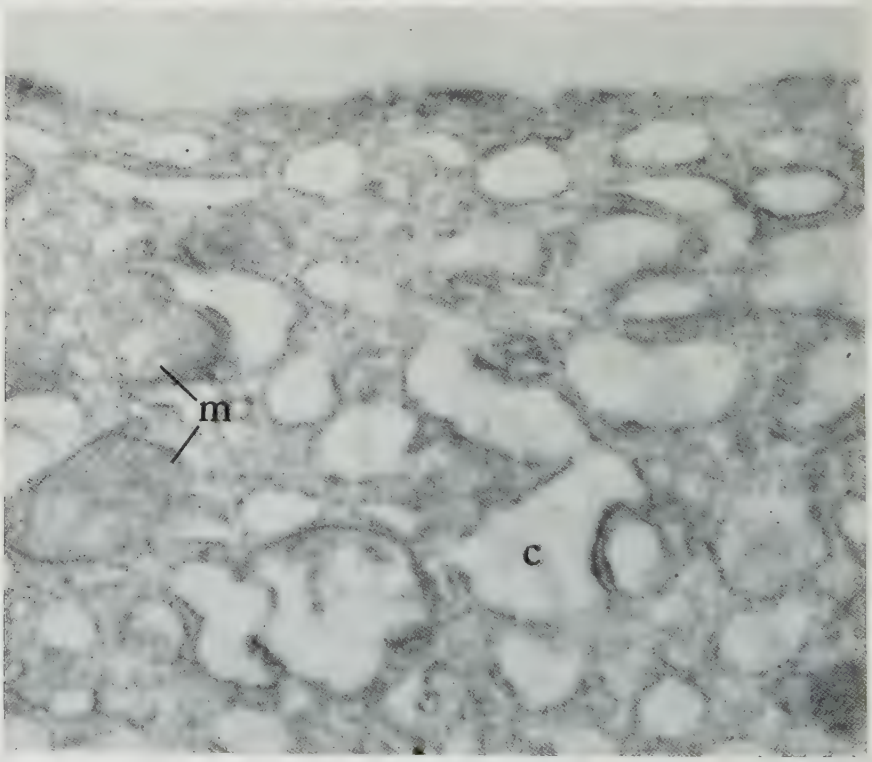

Fig. 8

Fig. 7. This electron micrograph illustrates a lamellar configuration of the endoplasmic reticulum of a rickety osteoblast with slight dilatations (*c*) between the smooth surfaces of these rough-surfaced components. Amorphous matrix material is visible in the upper right corner. Mitochondria (*m*) lie in the cytoplasm between the roughsurfaced boundaries of the cisternae (× 37 000)

Fig. 8. This electron micrograph illustrates an irregular pattern of the cisternae (*c*) which may be either the result of the plane of section or may be a real difference in the disposition of the components of the endoplasmic reticulum. Mitochondria appear at *m* (× 34000)

procollagen-collagen system we do not know what variety of substances may act as catalysts. The cisternae of the endoplasmic reticulum are the cellular compartments believed to be most likely responsible for the elaboration of the fibril precursor. This hypothesis is illustrated in Fig. 12 where a portion of osteoblast cytoplasm is depicted and a cisterna is shown open to the extracellular space, liberating the precursor of the collagen fibril; however, the precise mechanism whereby the cisternae come into continuity with the extracellular space has not been elucidated, therefore we leave a question mark within this area.

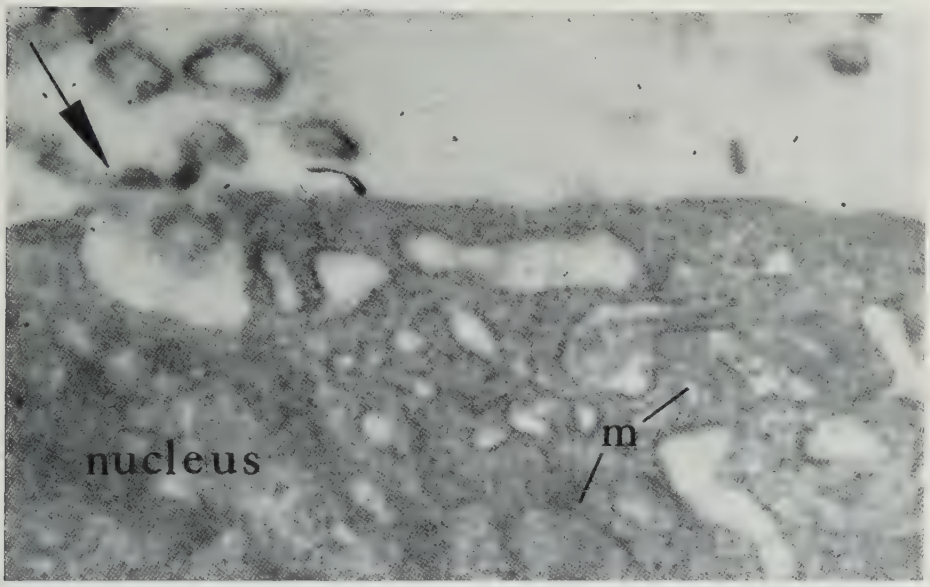

Fig. 9. This electron micrograph shows a portion of an osteoblast from a rickety rat and demonstrates a continuity between a cytoplasmic compartment and the extracellular space (arrow). The nucleus and mitochondria (*m*) are recognizable and the rough-surfaced components of the endoplasmic reticulum do not appear remarkable (× 30000)

Discussion

The relationship of ribonucleoprotein in cells to protein synthesis has been the subject of much speculation and many reports (Brachet 1945, Caspersson 1947, 1950, Thorell 1947). Correlated cytochemical and electron microscopic investigations have shown that the basophilic components of the cytoplasm consist principally of ribonucleic acid (Palade 1956, Siekevitz and Palade 1958, 1959), and in the present communication we have commented on the fine structure of the cytoplasmic elements of the osteoblast which are now regarded as responsible for cytoplasmic basophilia; these are the small granules (Palade particles, 1955) and the particles attached to membranes [referred to variously as rough-surfaced elements of the endoplasmic reticulum (Palade 1956), granular reticulum (Palay and Palade 1955), ergastoplasmic membranes (Bernhard and Roullier 1956), or alpha cytomembranes (Sjöstrand 1956)].

Recent reports have drawn attention to the importance of nucleic acids in the synthesis of peptide linkages. Berg (1958) has postulated the existence of adenyl amino acid as an intermediate in the synthesis of protein, and Zamecnik

and his co-workers (HOAGLUND et al. 1956, LITTLEFIELD et al. 1955, ZAMECNIK et al. 1957, 1958) have related the endoplasmic reticulum to protein synthesis by way of a soluble ribonucleic acid. Correlated biochemical and cytological investigations, such as those of SIEKEVITZ and PALADE (1956, 1958, 1959), on

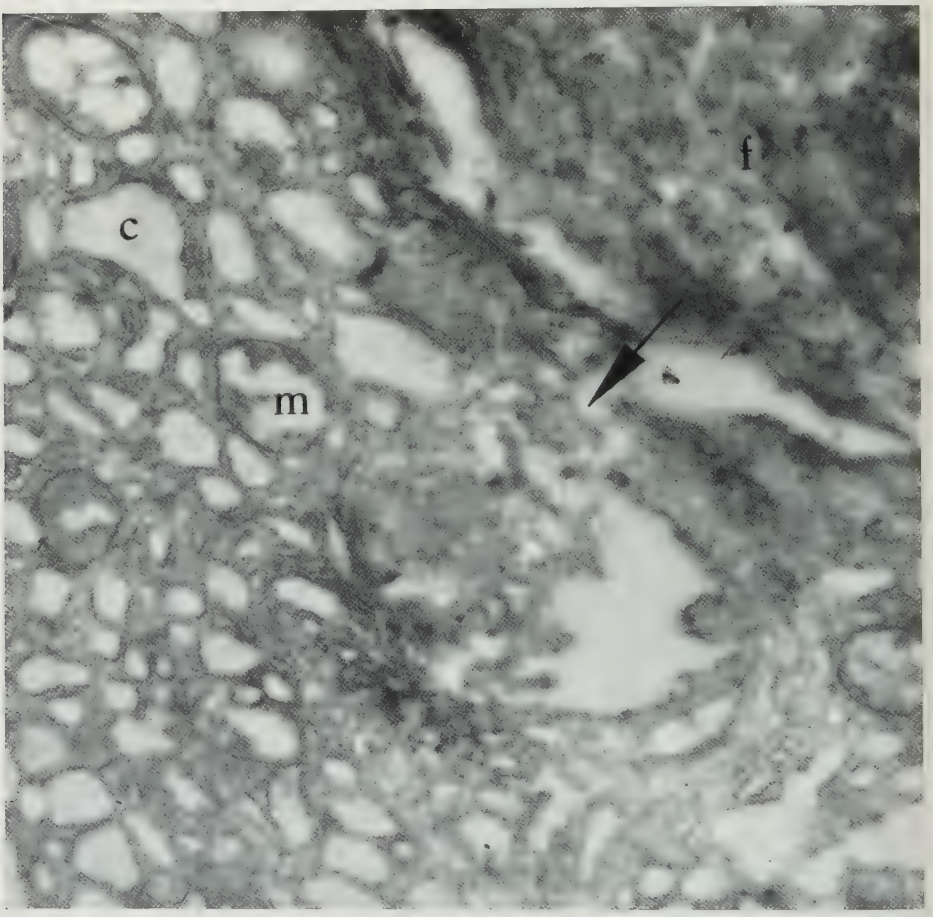

Fig. 10. This electron micrograph shows a portion of another osteoblast from a rickety rat in which collagenous matrix material can be seen within an indentation in the cytoplasm (arrow). This bay or inlet might be equated with such a continuity between the cytoplasm and the extracellular space, however the resolution achieved here does not allow a clear-cut interpretation to be made. The collagenous matric fibrils appear at *f*; mitochondria (*m*) and the cisternae of the endoplasmic reticulum (*c*) can be recognized

the pancreas have related the rough-surfaced component of the endoplasmic reticulum to the elaboration of protein by the pancreatic acinar cell.

As a system for the investigation of protein synthesis in cells the pancreas has served admirably, however the problem of identifying the end product remains. It is difficult to identify the specific secretion products of the pancreatic acinar cell when the material has been liberated from the cell, and difficult to distinguish one secretory product (chymotrypsinogen or lipase ?) from another. For this reason we felt that collagen, because of its characteristic fine structure,

might be more readily identified as a particular protein at the point where it is elaborated by the cell. This feature of being able to recognize a protein by its fine structure suggested that the osteoblast would represent a good model in which to observe the mechanism whereby protein is liberated from a cell. Our

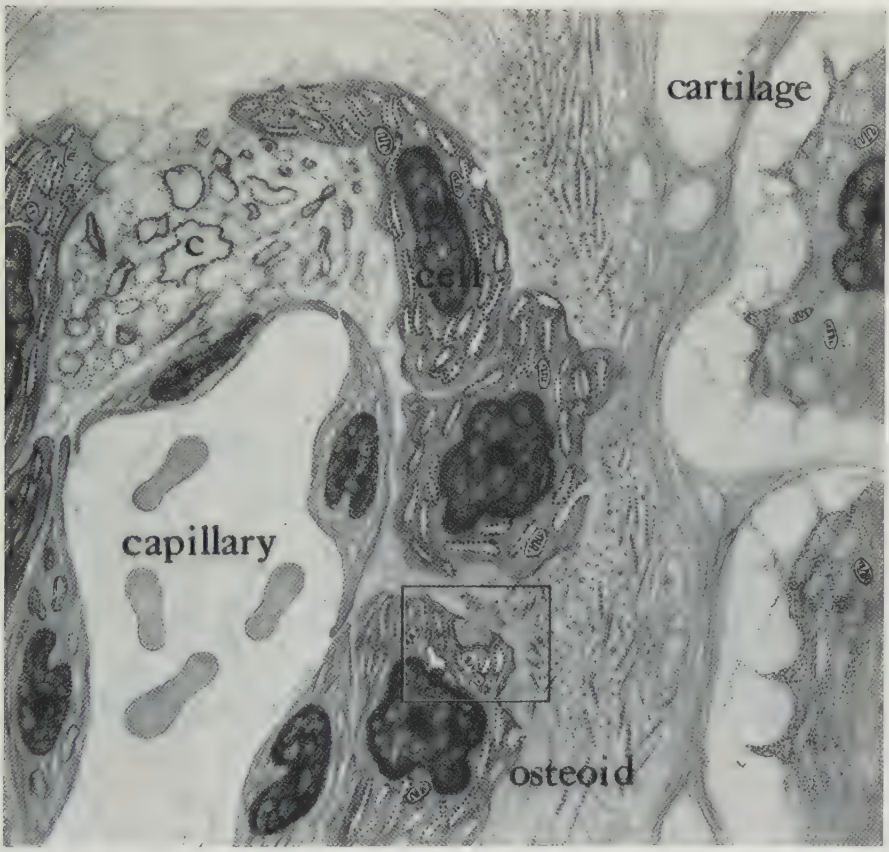

Fig. 11. This schematic drawing illustrates a composite picture of the cell-matrix relationships at the epiphyseal plate in rickets. No calcification of either cartilage or bone matrix is portrayed here, but it is reasonable to suggest that the general relationships between the small blood vessels, osteoblasts, bone matrix, cartilage matrix and cartilage cells would be the same if calcification had occurred. The difference between the structure of bone and cartilage matrix can be seen at the right where the bone matrix lies in opposition to the more delicate fibrils of the cartilage matrix. The difference in the fine structure of chondrocyte and osteoblast cytoplasm is illustrated. Within the square is a portion of an osteoblast which is shown at a higher magnification in Fig. 12

interest in this problem stemmed from a desire to confirm the suggestion that proteins are synthesized in a particular compartment of the cytoplasm and secreted therefrom.

With regard to the problem of the synthesis of collagen in particular, PORTER and PAPPAS (1959) concluded from their studies that the unit fibrils of collagen may form in close association with the cell surface. They remarked that collagen fibrils were never observed within the cell, a finding which we can confirm from the present studies and from other observations on bone cells under various conditions. Because of the alignment and particular orientation of fibrils so often

seen in studies on collagen, PORTER and PAPPAS suggest that some mechanism may affect the polymerization of the fibrils from the proteins available at the cell surface and produce the preferred orientation of these fibrils. Their suggestion is that there may be some peculiar area of the cell surface membrane which causes the orientation of subsequently developed collagen fibrils. In the experiments of PORTER (1949) and those of earlier authors there was evidence put forward to indicate that collagen fibrils close to cells were smaller in diameter than the collagen fibrils lying at some distance.

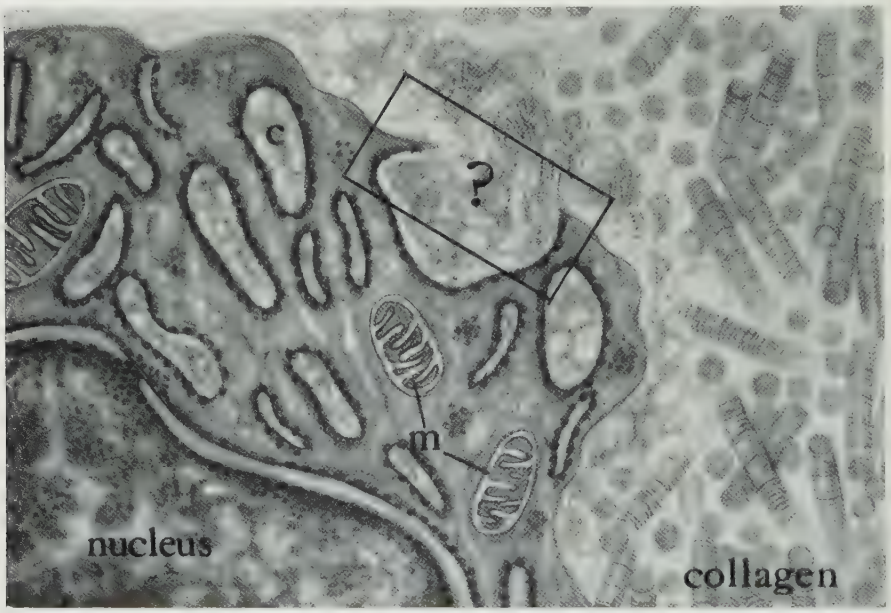

Fig. 12. This schematic drawing illustrates a cellular mechanism which may explain the relationship between the osteoblast cytoplasm and bone matrix. The osteoblast cytoplasm consists of abundant "rough-surfaced elements of the endoplasmic reticulum" or particle-covered membranes which enclose cisternae (*c*) or compartments within the cytoplasm. The relationship between these membranes (which with the attached particles consist largely of ribonucleoprotein) and the synthesis of protein is outlined in the discussion. This drawing suggests that the osteoblast may elaborate an afibrillar precursor of the collagen fibril; it is suggested that this material is synthesized within the cisternae of the endoplasmic reticulum and is liberated into the extracellular space in such a manner as illustrated here. Once the afibrillar precursor of collagen is liberated into the extracellular space it may polymerize to form the fibrils of collagen with their characteristic banding. The precise mechanism whereby the membrane-limited cisternae of the endoplasmic reticulum come into continuity with the cell surface membrane and thereby liberate their contents into the extracellular space remains in question

SYLVIA FITTON JACKSON has demonstrated that in avian tendon the collagen fibrils of developing bone increase in diameter while there is a relative decrease in the amount of interfibrillar material which surrounds the fibrils (1956, 1957). She concludes that this material contains collagen molecules which are deposited onto the growing fibrils in the form and packing appropriate to the characteristic fibre diagram of the collagen protein. This may be interpreted as evidence for further aggregation and growth of the more mature collagen fibril. This also might be interpreted as evidence that the collagen fibrils which have already been laid down could serve as a template for the fibrils which were yet to come.

These findings bring us to a consideration of the evidence which is available regarding the possible origins of newly formed collagen.

On the basis of extraction studies D. F. Jackson (1960) concludes that "at any given time in developing connective tissue there is a continuous spectrum of collagen aggregates of varying degress of strength of cross linkage dependent on the time that has elapsed since their constituent molecules were synthesized". This suggestion puts the observations of Orekhovitch (1957) and Gross (1954) on "procollagen" and "tropocollagen" into perspective and would seem to explain the present authors inability to visualize a direct relationship between the cytoplasmic constituents and the extracellular collagen fibrils. Recent evidence to support this hypothesis, that the precursor to the collagen fibril is elaborated within a particular and particulate component of the osteoblast cytoplasm, has been put forward by Lowther and Green (quoted in Jackson, 1960) who have found a collagen fraction which may be extracted with 0.14 m NaCl from the microsomal fraction of cells. Jackson suggests that this fraction probably represents the most recently synthesized collagen. This may be interpreted as further evidence in support of the general hypothesis linking cellular ribonucleoprotein to protein synthesis, and the scheme put forward in the contemporary literature (Zamecnik 1958). The difficulty of demonstrating a direct relationship between the endoplasmic reticulum and the synthesis of protein may be increased in the present experimental model because the rickety animal is growing slowly and therefore may be elaborating collagen at a slower than usual rate. Our inability to demonstrate more clearly a relationship in the present system should not, however, vitiate the acceptibility of the hypothesis as it is presented here, and we believe that by appropriate manipulation of the osteoblast—collagen system we may be able to further clarify the relationship between the osteoblast cytoplasm and the constituents of the extracellular matrix, and demonstrate the morphologic relationships between the various cytoplasmic compartments and the cell surface membrane and thereby further elucidate the intimate relationships between cells and their secretory products.

Summary

In an electron microscope study of the cellular components of the uncalcified epiphyseal cartilage and bone which is found in experimental rickets, the authors found that the osteoblasts and osteocytes did not differ in any remarkable way from osteoblasts and osteocytes of normal bone. The disposition and appearance of the endoplasmic reticulum in the rickety cells is compared with the constituents of the cytoplasm in cells from normal bone. An extracellular afibrillar material can be seen in the matrix in the immediate vicinity of many osteoblasts in addition to the collagenous fibrils of the uncalcified bone matrix (rickety osteoid). The authors propose an interpretation which may explain the origin of this afibrillar material and in addition may elucidate the relationship between the cells of this particular connective tissue and the extracellular matrix.

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(Direktor: Prof. Dr. med. et phil. E. Horstmann)

ELEKTRONENMIKROSKOPISCHE UND HISTOTOPOCHEMISCHE UNTERSUCHUNGEN ÜBER STRUKTUR UND BILDUNG DER ZONA PELLUCIDA MENSCHLICHER EIZELLEN

Von

H.-E. Stegner und H. Wartenberg

Mit 7 Textabbildungen, davon 2 farbigen

(Eingegangen am 7. November 1960)

Eitransportmechanismus, Befruchtungs- und Implantationsmodus und andere Faktoren bestimmen die artverschiedenen Strukturen der tierischen Eihüllen. Metatheria und Eutheria — mit Einschränkungen auch die Protheria — bilden um die Oozyte im Verlaufe der Eizellreifung eine in Bau und Funktion vergleichbare Schutzhülle, die Zona pellucida. Ihre wichtigste Aufgabe besteht offenbar in einer zeitlich begrenzten Sicherung der frühen Entwicklungsabläufe des Eies. Wieweit sie echte Membranfunktionen mit der Fähigkeit der Ingestion und Permeation besitzt, ist bisher nur wenig bekannt. Immerhin läßt die morphologische Struktur der Zona pellucida Zweifel an einer völligen Isolierung und Autonomie der ersten Entwicklungsprozesse und Raum für Spekulationen über metabolische Beziehungen zum jeweiligen Nachbarsubstrat aufkommen. Die Zona pellucida der Eizelle der Säugetiere zeigt auf Grund der bisherigen licht- und elektronenmikroskopischen Befunde zahlreicher Autoren ein einheitliches Bauprinzip. Auch bei Ausdehnung der Betrachtungen auf die gesamten Vertebraten finden wir eine Reihe von Analogien, die allerdings mit Vorbehalt zu verwerten sind (Amphibien und Fische s. bei Arndt 1960; Dollander 1956; Kemp 1956a, b; Sterba 1957; Sterba u. Franke 1959; Wartenberg u. Gusek 1960). Ein grundsätzlicher Unterschied besteht schon insofern, als die Keime der Wirbeltiere in der Regel ihre definitiven Eihüllen erst in den weiblichen Geschlechtswegen erhalten. Eine Differenzierung der Eihüllen an den Orten der Eizellbildung findet sich vornehmlich bei den Säugern. Die Zona pellucida besteht aus einer extrazellulären amorphen Hüllsubstanz, deren Homogenität von radiär und schräg verlaufenden Fortsätzen der angrenzenden Follikelepithelzellen und von offenbar variablen Ausstülpungen (Mikrovilli) des Plasmalemm der Eizelle unterbrochen wird. Derartige interzelluläre Protoplasmabrücken zwischen Follikelzelle und Oozyte hat Kemp (1958) bei Repräsentanten aller Vertebratenklassen nachweisen können. Exakte Untersuchungen von Yamada u. a. (1957) am Mäuseovar, Trujillo Cénoz und Sotelo (1959) am Kaninchenovar und Sotelo und Porter (1959) sowie Odor (1960) am Rattenovar haben gezeigt, daß es sich bei diesen zytoplasmatischen „Anastomosen" nicht um unmittelbare synzytiale Plasmaverbindungen handelt, sondern lediglich um plasmatische Zellkontakte. Plasmalemm der Oozyte und Follikelzellmembranen sind in jedem Falle durch einen relativ konstanten Spaltraum getrennt. Wir konnten bei unseren Untersuchungen an menschlichen Eizellen entsprechende

Befunde erheben (Wartenberg und Stegner 1960). Zum Teil sind die Follikelzellfortsätze an der äußeren Zirkumferenz der menschlichen Oozyte mit charakteristischen Haftplatten (Desmosomen) verankert oder unter Wahrung eines trennenden Spaltraumes in das Zytoplasma der Eizelle eingegraben.

Wenngleich dank der Elektronenmikroskopie eine weitgehende Übereinstimmung der Ansichten über den relativ einheitlichen submikroskopischen Bau der Zona verschiedener Säuger erzielt wurde, so bestehen doch noch immer Diskrepanzen in der Frage nach der *Bildung* der Zona pellucida. Eine Reihe neuer Teilbefunde hat mehr Verwirrung als Aufklärung gebracht, letztlich aber wieder der schon von Waldeyer (1870) geäußerten Meinung einer uneinheitlichen Abstammung neuen Nachdruck verliehen. Nach wie vor ist unentschieden, ob es sich bei der Zona pellucida um eine heteromorphe Hüllsubstanz handelt, zu deren Bau Follikelzellen und Oozyte gemeinsam substantiell beitragen, oder ob einer der beiden Faktoren lediglich einen induktiven Einfluß auf die definitive Ausbildung der Zona ausübt.

Unsere anschließend dargestellten Befunde sind das Resultat parallel durchgeführter elektronenmikroskopischer und histotopochemischer Untersuchungen an menschlichen Eizellen verschiedener Reife.

Material und Methoden

Die elektronenmikroskopischen Untersuchungen wurden an 11 menschlichen Eizellen durchgeführt. 10 Eizellen entstammen Tertiärfollikeln mittlerer Größe (3—8 mm), eine Eizelle aus einem Sekundärfollikel. Bei dem Untersuchungsgut handelt es sich ausschließlich um Operationsmaterial. Tubargraviditäten, Ovarialzysten und Collumkarzinome bildeten die Operationsindikation. Das Alter der Patientinnen lag zwischen 18 und 26 Jahren. Bei der Präparation der Ovarien kamen zwei Methoden zur Anwendung. Ein Teil der Follikel wurde in toto aus dem Ovarialstroma herauspräpariert, segmentiert und in einer isotonen (NaCl) und auf p_H 7,2 gepufferten (Veronal-Azetat) 1%igen OsO_4-Lösung bei 4° C, 2 Std lang fixiert. Das Material wurde danach in üblicher Weise in 70%igen Alkohol gebracht. Anschließend wurde der Cumulusbezirk unter dem Lupenmikroskop herausgeschnitten. Die aufgefundenen Eier mit umgebenden Follikelzellen des Cumulus oophorus wurden entwässert und über Azeton in Methyl-Butylmethacrylat 1:3 eingebettet. Ein zweites Verfahren zur Gewinnung von Eizellen bestand in der Follikelpunktion nach den Angaben von Shettles (1957, 1960). Der Einstich erfolgt dabei in das Ovarialstroma in einiger Entfernung vom Follikel; die Nadel wird unter der Oberfläche durch das Stroma an die angrenzenden Follikel herangeführt. Der Sekundärfollikel wurde durch Stufenschnitte im rindennahen Ovarialbezirk aufgefunden. Zur Untersuchung kam nur einwandfrei durchfixiertes Material. Die Ultradünnschnitte wurden teils auf einem Porter-Blum-Mikrotom, teils auf einem Leitz-Mikrotom (Fernández Morán) hergestellt, die Aufnahmen mit einem Siemens Elmiskop I bei 80 kV gemacht. Plattenmaterial: Perutz-Kontrast und Elektroguil (Guilleminot, Paris).

Zu histochemischen Untersuchungen wurden die zunächst gröber unterteilten Ovarien in neutralem 5%igem Formol fixiert und in üblicher Weise in steigenden Alkoholkonzentrationen entwässert. Danach erfolgte bei einem Teil des Materials Aufhellung in Cedernholzöl und über Chloroform die Einbettung in Paraffin. Folgende histochemische Reaktionen, vorwiegend zum Nachweis und der Differenzierung von Kohlenhydraten bestimmt, kamen zur Anwendung: die Perjodsäure-Schiff-Reaktion nach McManus (1946) in Verbindung mit Speicheldigestion und reversibler Azetylierung nach Lillie (1954, S. 160) sowie die Färbung mit Bestschem Karmin. Durch Prüfung der Toluidinblaumetachromasie (Pearse 1954, S. 432) bei p_H 4 in Walpole-Puffer und Anwendung der kolloidalen Eisenbindungsreaktion nach Hale in der Modifikation von Müller (1955, 1959), vor und nach Methylierung, wurden saure Mukopolysaccharide dargestellt. Daneben erfolgte Färbung mit Alcianblue und Astrablau sowie Kombination der PJS- und Haleschen Reaktion nach

Ritter u. Oleson (1956). Zum Lipoidnachweis dienten die Sudanschwarzfärbung, die Färbung mit Nilblausulfat und der Hämateintest nach Baker (1946). Zur Darstellung maskierter Fette wurde die Fluoreszenzmethode mit Benzpyren-Koffein nach Berg (1951) angewandt.

Elektronenmikroskopische Befunde

Die Grundsubstanz der Zona pellucida bietet elektronenmikroskopisch in allen Entwicklungsphasen ein homogenes, strukturell bis auf geringe Dichteunterschiede relativ einförmiges Bild, obgleich sie ein komplexes Gemisch aus

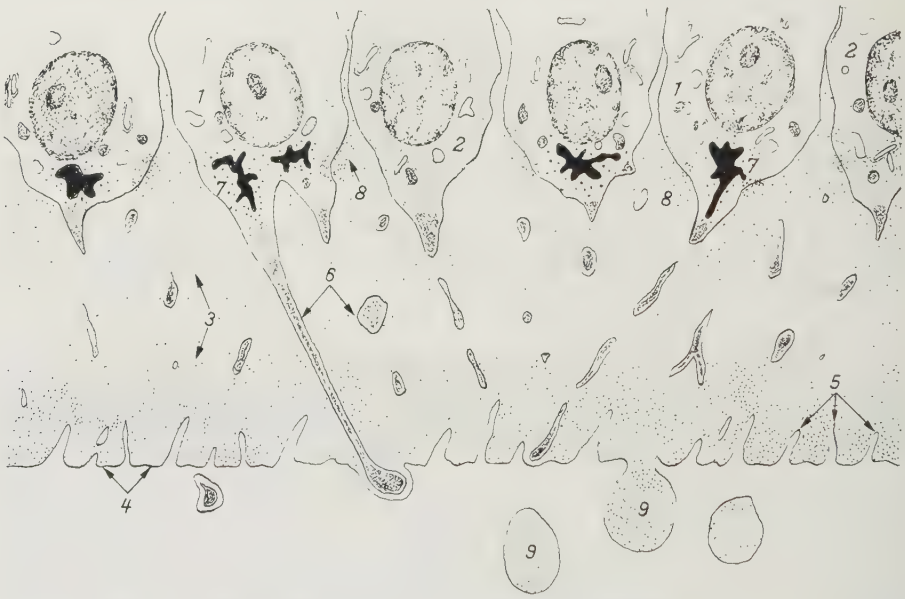

Abb. 1. Schema der morphologischen Beziehungen zwischen Eioberfläche, Zona pellucida und Follikelepithel einer menschlichen Oozyte. *1* „Helle" Follikelzellen; *2* dunkelgranulierte Follikelzellen; *3* Zona pellucida mit konsolidierten zentralen und lockeren peripheren Anteilen; *4* Plasmalemm; *5* Mikrovilli; *6* Fortsätze der Follikelzellen mit granulärem Inhalt; *7* bizarr verzweigte grobgranuläre Konzentrate (saure Mukopolysaccharide); *8* körnige intra- und extrazelluläre Verdichtungsbezirke (Sekretionsorte?); *9* paraplasmatische Einschlüsse mit einem, der Zonagrundsubstanz strukturanalogen Inhalt

Verbindungen der verschiedensten Stoffklassen darstellt. Im Sekundärfollikel imponiert sie als eine schmale, konzentrisch um das Ei angeordnete Hüllschicht von homogener, wolkiger Struktur. Die Follikelzellen sind dicht aufgeschlossen und bilden dadurch eine relativ scharfe periphere Begrenzung. Zytoplasmafortsätze sind in reichlicher Anzahl vorhanden und durchziehen in schräger, beinahe tangentialer Richtung die Grundsubstanz der Zona pellucida. Sie enthalten dichtes granuläres Material. Dieses Material konzentriert sich an den Abgangsstellen der Fortsätze und verschiedentlich in den Randbezirken der Corona radiata-Zellen. Die Zellgrenzen sind in alternierenden Bezirken verwaschen; es besteht der Eindruck einer Emission von granulärem Material in Richtung auf die Zona oder die angrenzenden Interzellularspalten der Follikelzellen. Man erkennt zwei Haupttypen von Follikelzellen: einen helleren Typ mit sehr lockerem Zytoplasma und einen dunkleren, dicht granulierten Typ. Es läßt sich zunächst nicht entscheiden, ob es sich dabei um Funktionszustände

oder Degenerationszeichen handelt. Die zentrale Begrenzung der Zona bildet das Plasmalemm der Oozyte, welches sich in diesem Stadium bereits mit zahl-

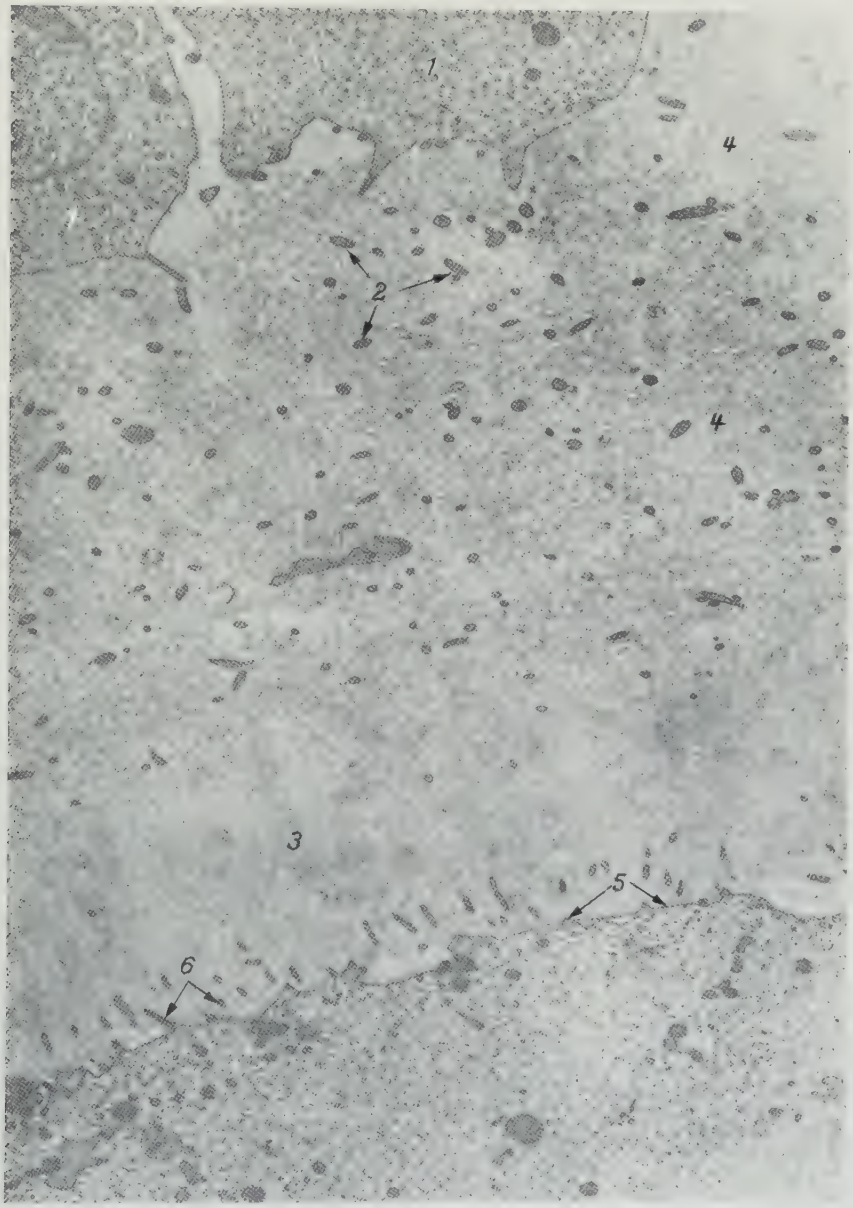

Abb. 2. Ausschnitt aus der Zona pellucida eines menschlichen Ovarialeies (Tertiärfollikel). *1* Follikelzellen; *2* Anschnitte von Fortsätzen der Follikelzellen; *3* zentrale konsolidierte Anteile der Zona pellucida; *4* peripheres aufgelockertes Material der Zona pellucida; *5* Plasmalemm; *6* Mikrovilli. Vergr. 10000 ×

reichen Mikrovilli in die Zonagrundsubstanz einstülpt. Eine Verbreiterung der Zona und Dichteunterschiede der Grundsubstanz sind die wesentlichsten Veränderungen, die mit der Entwicklung zum Tertiärfollikel auftreten.

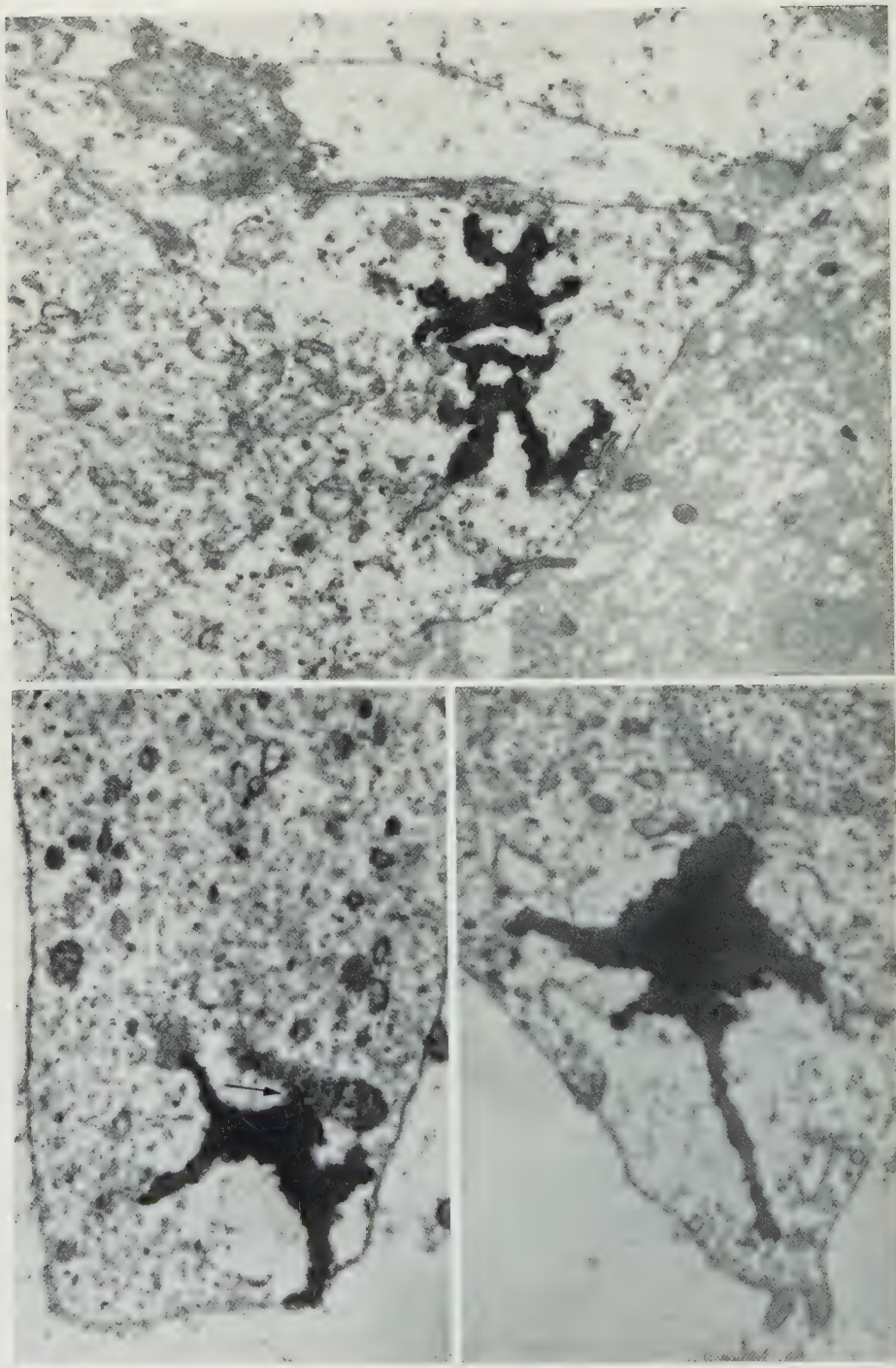

Abb. 3a—c. Ausschnitte aus Follikelzellen der Corona radiata eines menschlichen Tertiärfollikels. Im Zytoplasma dichte, bizarr verzweigte, grobgranuläre Konzentrate (saure Mukopolysaccharide). Bei ↑ (b) unmittelbare morphologische Beziehungen zu einem Mitochondrium. Ausstreuung grobpartikulären Materials in die Umgebung der Konzentrate. Vergr. 17000 ×

Die nachfolgend dargestellten histochemischen Veränderungen als Ausdruck nicht unerheblicher chemischer Umbauvorgänge treten im morphologischen Bilde nicht zutage. Abb. 1 schematisiert den submikroskopischen Aufbau der Zona pellucida eines Tertiärfollikels. Die amorphe Grundsubstanz ist im zentralen Drittel deutlich verdichtet (Abb. 2). Nach der Peripherie zu erkennt man eine Auflockerung des Materials, welches sich in die Interzellularspalten der Follikelzellen fortsetzt. Auch hier finden sich stellenweise kleine Verdichtungsbezirke unmittelbar gegenüber intrazytoplasmatischen granulären Konzentrationen der Follikelzellen (Sekretionsvorgänge ?).

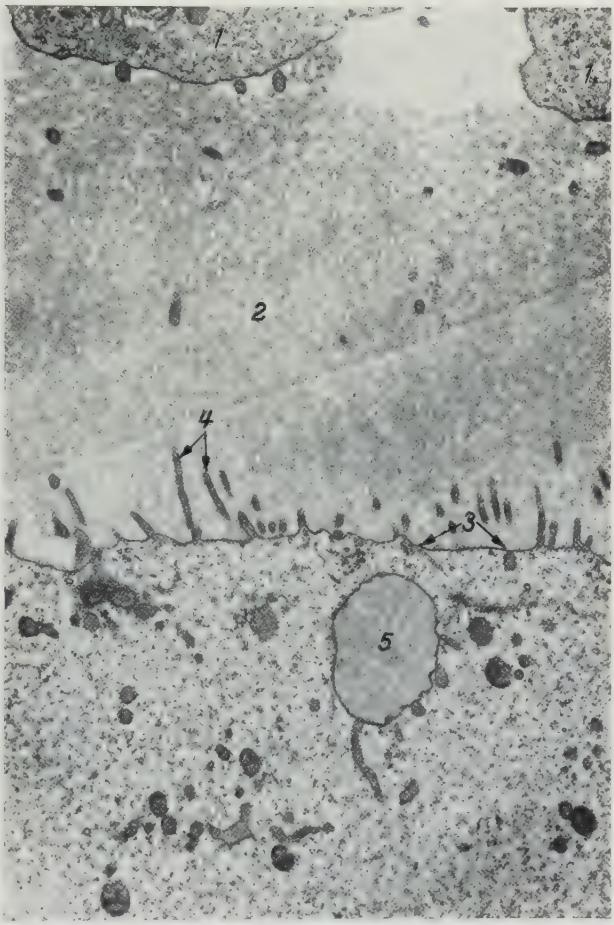

Abb. 4. Menschliche Oozyte. Ausschnitt aus der Zona pellucida und Eioberfläche. *1* Follikelzellen; *2* Grundsubstanz der Zona pellucida; *3* Plasmalemm; *4* Mikrovilli; *5* Vakuole (Durchmesser 1,0 × 1,5 μ), deren Inhalt in Struktur und Dichte dem Zonamaterial gleicht (paraplasmatisches Material zum Aufbau der Zona ?). Vergr. 14000 ×

Einen auffallenden Befund bilden große, auch lichtmikroskopisch nachweisbare, bizarr verzweigte und sehr dichte Substanzen innerhalb der basalen Bezirke der Follikelzellen (Abb. 3a—c). Nach unserer Meinung handelt es sich dabei in Übereinstimmung mit histochemischen Befunden um Ansammlungen saurer Mukopolysaccharide.

Elektronenmikroskopisch besteht eine geringe Ähnlichkeit dieser Substanzen mit Lipoideinschlüssen. Beiden gemeinsam ist die starke optische Dichte. Im Gegensatz zum submikroskopischen Bau der Lipoideinschlüsse findet sich hier eine grobpartikuläre Grundstruktur und eine stärker auffällige Verästelung. Grobgranuläres Material ist in die unmittelbare Umgebung dieser Gebilde verstreut, während die Granulierung der Zellfortsätze von feinkörniger Beschaffenheit ist.

Wenn die erhobenen Befunde eine substantielle Beteiligung der Follikelzellen an der Zonabildung erneut sehr wahrscheinlich machen, so finden sich doch auch wieder Beobachtungen, die für eine aktive Mitwirkung der Oozyte selbst

an diesem Bildungsprozeß sprechen könnten. (Die Schwierigkeit der Ableitung dynamischer Vorgänge aus morphologischen Zustandsbildern bedarf keiner besonderen Betonung.) Unmittelbar unter dem Plasmalemm finden sich in den Randbezirken der Oozyte Vakuolen mit einem Durchmesser von etwa 1,0—1,5 μ, deren Inhalt in Struktur und Dichte dem Material der Zona pellucida völlig gleicht (Abb. 4). In einzelnen Fällen waren derartige Vakuolen in den perivitellinen Spalt geöffnet (Abb. 5), und ausgestoßenes Material lag in flacher

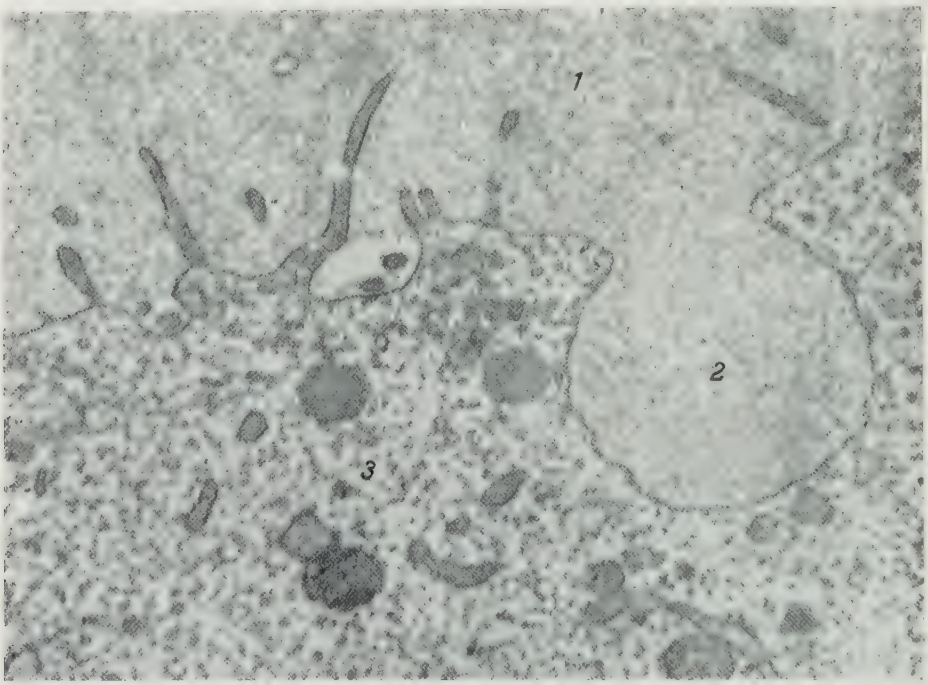

Abb. 5. Randvakuole einer Oozyte in offener Verbindung zur Zonagrundsubstanz. *1* Zona pellucida; *2* in die Zona geöffnete Vakuole; *3* Ooplasma. Vergr. 30000 ×

Ausbreitung unmittelbar unter den konsolidierten Anteilen der Zona pellucida. Schichtungsphänomene als Ausdruck eines appositionellen Wachstums der Zona waren nicht eindeutig nachweisbar.

Histochemische Befunde

In den Interzellularspalten des Follikelepithels zweischichtiger, spätestens dreischichtiger Sekundärfollikel treten ubiquitär homogene Substanzen auf, die eine schwach positive PJS- und eine starke Eisenbindungsreaktion zeigen (Abb. 6). Sie färben sich mit Alcianblue und Astrablau kräftig an und geben mit Toluidinblau eine auch nach Alkoholbehandlung weitgehend stabile, metachromatische Reaktion, die bei vorausgegangener Methylierung negativ ausfällt. Diese Substanzen, bei denen es sich offenbar um saure Mukopolysaccharide handelt, liegen vorwiegend interzellulär. Darüber hinaus erkennt man saures Material in den Randabschnitten der Granulosazellen mit dichter Konzentration in den zipfligen Ausstülpungen der Zellen und am Abgang der Zellfortsätze.

Diese Anordnung sieht man besonders deutlich bei beginnender Antrumbildung an dissoziierten und isolierten Follikelzellen. An der äußeren Zirkumferenz des Eies konfluieren diese Substanzen und ordnen sich konzentrisch um die Oozyte.

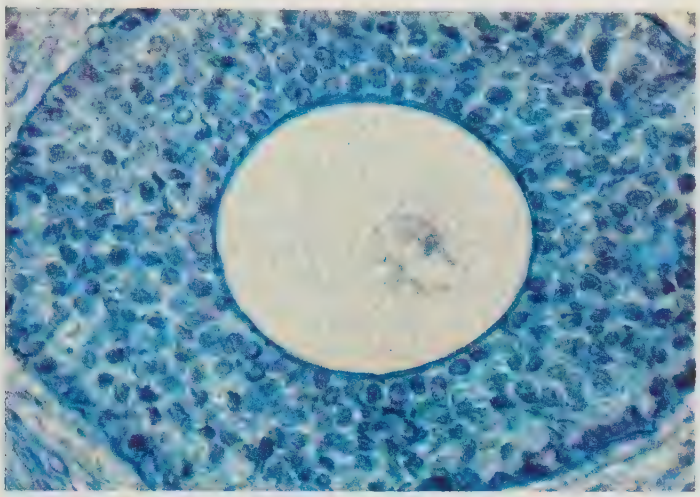

Abb. 6

Abb. 7

Abb. 6. Menschliche Eizelle im Stadium des Sekundärfollikels. Darstellung saurer Mukopolysaccharide mit Astrablau. Kräftige gleichmäßige Färbung der Zona pellucida und homogener Substanzen ubiquitär in den Interzellularspalten der Follikelzellen. Kodachrom, Vergr.: 175 ×

Abb. 7. Menschliche Eizelle (Anschnitt außerhalb des Nukleus) im Stadium des Tertiärfollikels. Kombinierte PAS-Hale-Reaktion. Zweiteilung der Zona pellucida in einen zentralen stark PAS-positiven Bereich (rot) und eine äußere Hale-positive konzentrische Zone aus anionischen Polysacchariden (blau). Saure Substanzen finden sich auch in granulärer Form innerhalb der angrenzenden Follikelzellen und deren Fortsätzen. Kodachrom, Vergr. 175 ×

Sie stellen die erste Anlage einer interzellulären Hüllsubstanz dar, die allerdings noch nicht die charakteristische Dichte und Lichtbrechung der definitiven Zona pellucida aufweist. Mit zunehmender Verbreiterung dieser Hüllschicht tritt eine Änderung im histochemischen Verhalten ein. Die einahen Abschnitte der Zona konsolidieren sich, die PJS-Reaktion verstärkt sich in diesem Bereich und zeigt einen Intensitätsabfall nach der Peripherie. Saure Substanzen sind

in den verdichteten zentralen Anteilen der Zona nicht mehr nachweisbar. Sie finden sich jetzt lediglich im äußeren konzentrischen Bereich der Zona, in den angrenzenden Interzellularspalten der Corona-radiata-Zellen und in Form grober und feiner Granula im Zytoplasma der Follikelzellen und deren Zellausläufern. In kombinierten PJS-Hale- oder PJS-Alcianblue-Präparaten (Abb. 7) erkennt man eine deutliche Zweischichtung der Zona pellucida: einen zentralen tiefrot gefärbten PJS-positiven Bereich, welcher fließend in eine kräftig blau gefärbte, äußere konzentrische Zone übergeht, deren Dichte sich in der Peripherie auflockert. Die beiden farblich differenten Anteile sind nicht scharf gegeneinander abgesetzt, sondern zeigen einen allmählichen Übergang. Dadurch entsteht eher der Eindruck einer in zentripetaler Richtung ablaufenden Umlagerung (?) der sauren Mukopolysaccharide in neutrale PJS-positive Verbindungen, als der einer gegeneinandergerichteten appositionellen Ablagerung von Substanzen aus zwei voneinander unabhängigen Quellen.

Die bereits elektronenmikroskopisch aufgefundenen Indizien einer substantiellen Beteiligung der Oocyte an der Zonabildung lassen sich auch histochemisch nachweisen. Die Peripherie des Ooplasma verschiedener Eizellen im Stadium des Tertiärfollikels enthält kleine, der Zona benachbarte oder unmittelbar anliegende perjodreaktive Granula, die auf Grund ihrer Anordnung und Größe mit den im elektronenmikroskopischen Teil beschriebenen, der Zona strukturanalogen Vakuolen identisch sind. Lipoide waren mit keiner der im methodischen Teil aufgeführten Reaktionen innerhalb der Zona nachweisbar.

Diskussion der Befunde

Reifegrad und Aktivitätsphasen verändern laufend das morphologische und histochemische Bild der Zona pellucida. Dazu kommen Rückbildungsprozesse, die besonders in ihren frühen Stadien die Beurteilung physiologischer Differenzierungsvorgänge stören können. Untersuchungen eines großen Materials sind nötig, um in allen Reifungsstufen vorhandene Degenerationsformen von Entwicklungsphasen in ihren morphologischen Wandlungen korrekt zu trennen. Die aus der Literatur bekannten histochemischen Befunde an der Zona pellucida verschiedener Tierarten sind für sinnvolle vergleichende Betrachtungen noch zu vereinzelt. Bei der Ratte (Braden 1952), dem Kaninchen (Braden 1952, da Silva Sasso 1959) und beim Menschen (s. bei Watzka 1957) ist übereinstimmend die Kohlenhydratkomponente als wesentlicher Bestandteil gefunden worden. Der Kohlenhydratanteil bildet offenbar die prosthetische Gruppe innerhalb eines Glykoproteids. Anionische Polysaccharide stellen einen, vermutlich in Abhängigkeit vom Entwicklungsgrad, variablen Anteil. da Silva Sasso (1959) beschreibt beim Kaninchen einen schwächeren Ausfall der Haleschen Eisenbindungsreaktion in den zentralen Anteilen der Zona, dagegen eine stark positive Reaktion in den peripheren Abschnitten. Wir haben saure Mukopolysaccharide als primäres Substrat der Zona pellucida in Sekundärfollikeln nachweisen können und bei voll ausgebildeter Zona des Tertiärfollikels eine Gliederung in einen stark PJS-positiven, zentralen konzentrischen Bereich ohne saure Gruppen und einen, ohne scharfe Grenze anschließenden, äußeren zirkulären Teil aus anionischen Polysacchariden gefunden. Über eine Abstammung der sauren Mukopolysaccharide von den Follikelzellen bestehen kaum noch Zweifel. Aktivitäts- und Ruhe-

phasen wechseln im Aufbau der Zona offenbar ebenso wie im Wachstum der Follikel. Beide Prozesse müssen nicht synchron ablaufen. Im Ruhezustand ist die Zona in ihrer ganzen Breite weitgehend konsolidiert. Innen- und Außenrand sind relativ scharf begrenzt. Die Follikelzellen sind dicht aufgeschlossen. Histochemisch findet sich kein Anhalt für die Produktion und Abgabe nennenswerter Mengen an sauren Substanzen. Nur in einer sehr schmalen Außenzone lassen sich saure Mukopolysaccharide nachweisen. Vorausgesetzt, daß man den Granulosazellen zumindest einen Anteil an der Bildung der Zona pellucida zuerkennt, entspricht eine vermehrte, gerichtete Abgabe saurer Mukopolysaccharide offenbar einem Stadium gesteigerter Neubildungs- und Aufbauvorgänge. In diesen Fällen sind die Zellen der Corona radiata von der Zona abgerückt, und der mit reichlich lockerem Hale-positiven Material angefüllte Spaltraum wird von den plasmatischen Follikelzellfortsätzen überbrückt, welche in die Zona einstrahlen. Die Priorität der sauren Polysaccharide und ihr kontinuierlicher Übergang in den sekundär auftretenden zentralen perjodatpositiven Bereich der Zona pellucida, sprechen für eine chemische Umwandlung der anionischen Polysaccharide nach ihrer Anlagerung an die Peripherie der Oozyte.

Der Modus dieser Umwandlung, die mit Verlust oder Bindung der sauren Gruppen (Uronsäuren, Estersulfat) einhergeht, ist nicht bekannt. Ob die PJS-positiven Einschlüsse in den Randbezirken der Oozyte tatsächlich paraplasmatische Baustoffe zur Zonabildung darstellen, läßt sich nicht entscheiden. Strukturelle Homologie bedeutet noch nicht gleiche Herkunft. Die endgültige Deutung dieser Einschlüsse steht noch aus. Es ist unwahrscheinlich, daß sie lediglich Degenerationsprodukte sind, da wir sie in unserem Untersuchungsgut gleichhäufig sowohl in sehr gut erhaltenen Oozyten, als auch in Eizellen mit den Zeichen beginnender Atresie finden konnten.

Summary

Examined with the electron microscope the zona pellucida of human oocytes represents an extracellular, amorphous substance with slight differences in density. There is a greater consolidation in the inner parts and a drecrease of density towards the periphery. The plasmalemma of the oocyte forms a large number of slender projections (microvilli) penetrating the homogeneous groundsubstance of the zona pellucida. Plasmatic elongations of the follicle cells extending towards the oocyte traverse the zona in oblique or tangential directions and end at the oocytes surface forming a contact relationship (partially characteristic desmosomes). No syncytial communication between ooplasma and follicle cell cytoplasm can be demonstrated. The follicle cell processes contain finely granular material. The cytoplasm of numerous follicle cells facing the oocyte containes branched deposits of compact and dense substances with a granular groundstructure. Histochemically these substances react like anionic polysaccharides. Alternating zones of the follicle cell membranes show vaguely outlined lesser density. Intra- and extraplasmatic granular concentrations in these areas probably represent secretion processes. Acid mucopolysaccharides are the primary substrat of the zona pellucida whose deposition begins almost in the state of a bilaminar secondary follicle. Possibly this material is incorporated in the definite complex of glycoproteins by means of loss or binding of the acid

groups (uronic acids, ester sulphates). PAS-positive inclusions about 1,0 to 1,5 μ in diameter lying in the cytoplasm of the follicle cells immediately adjacent to the plasmalemma or in open communication with the perivitelline space, represent probably paraplasmatic components for the building of the zona. Studied with the electron microscope these inclusions resemble strikingly the amorphous and cloudy ground substance of the zona pellucida. Stratified structures which would embody an appositional growth of the zona have not been demonstrated.

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ETUDE PRELIMINAIRE, AU MICROSCOPE ELECTRONIQUE, DE L'EMINENCE MEDIANE DU COBAYE*

Par

J. Barry et G. Cotte

Avec 6 Figures dans le Texte

(Manuscrit reçu le 7 Novembre 1960)

Introduction

La zone de contact proximale adéno-neurohypophysaire représente l'un des dispositifs essentiels d'articulation de la préhypophyse et de l'hypothalamus et, à ce titre, a fait l'objet d'assez nombreux travaux neurohistologiques, en particulier chez les Mammifères (Vasquez-Lopez 1942; Spatz, Diepen et Gaupp 1948; Diepen 1950; Rumbaur 1950; Nowakowski 1950, 1951; Spatz 1951, 1958; Knoche 1953; Bretschneider 1953, 1956; Metuzals 1954; Hanström 1954, 1955; Hagen 1953, 1955, 1957; Stutinsky 1955, 1957; Becker 1955; Diepen et Engelhardt 1958; Green et Maxwell 1959; Martinez 1960; Barry 1960; Barry et Lenys 1960). Par contre, la plupart des recherches microélectroniques relatives à l'appareil hypothalamo-hypophysaire concernent la «voie neurosécrétoire» hypothalamo-hypophysaire ou le lobe nerveux de l'hypophyse (Palay 1955, 1957; Green et van Breemen 1955; Duncan 1955, 1956; Barry 1957; Bargmann et Knoop 1957; Bargmann, Knoop et Thiel 1957; Fujita 1957; E. et H. Legait 1957, 1958; Bargmann 1958; Bretschneider 1958; Hartmann 1958; Green et Maxwell 1959; Gerschenfeld, Tramezzani et de Robertis 1960; Bargmann et Knoop 1960; de Robertis 1960). A quelques exceptions près (Palay 1957; Bretschneider 1958; Green et Maxwell 1959) la structure fine de la région infundibulaire ne semble pas avoir retenu l'attention et, lorsqu'elle a été étudiée incidemment, aucune mention particulière ne paraît avoir été faite de l'important problème des connexions neuro-vasculaires au niveau de l'éminence médiane. Nous nous proposons de rapporter ici les premiers résultats de nos recherches sur la structure fine de l'éminence médiane du cobaye. Nous espérons pouvoir compléter ces résultats ultérieurement et les étendre à d'autres espèces de Mammifères.

Matériel et technique

Nous avons utilisé des cobayes de 3 à 400 grammes, sacrifiés par décapitation après anesthésie sous cloche à l'éther. Les cerveaux ont été disséqués rapidement et extraits de la boîte cranienne. Quelques gouttes de solution tamponnée d'acide osmique à 2% ont été déposées sur la région infundibulaire. Cette dernière a été disséquée, sous le contrôle de la loupe binoculaire, et l'éminence médiane isolée entre deux traits de rasoir, l'un au niveau de sa zone d'insertion sur le tuber cinereum, l'autre au niveau de sa zone de transition avec la tige proprement dite. Les pièces, fixées une heure et demie dans OsO_4 à 2%, tamponné

* Avec la collaboration technique de Madame G. Cotte.

à p_H 7,3 selon Palade, ont été déshydratées puis incluses dans du méthacrylate de butyle additionné de 10% de méthacrylate de méthyle. Les blocs ont été ensuite débités au microtome de Porter-Blum, en coupes ultrafines alternant avec des coupes semi-fines de contrôle. Les coupes ultrafines, recueillies sur treillis munis de membranes supports de formvar, ont été examinées au microscope électronique RCA EMU 3 C. Les coupes semi-fines de contrôle (2 à 3 microns), utilisées pour le repérage, ont été examinées en contraste de phase au microscope binoculaire Wild muni du dispositif Varicolor de Locquin.

Observations personnelles

Nos observations ont porté essentiellement sur les fibres nerveuses myéliniques présentes au niveau de la zona fibrillaris sous-épendymaire, sur les fibres amyéli-

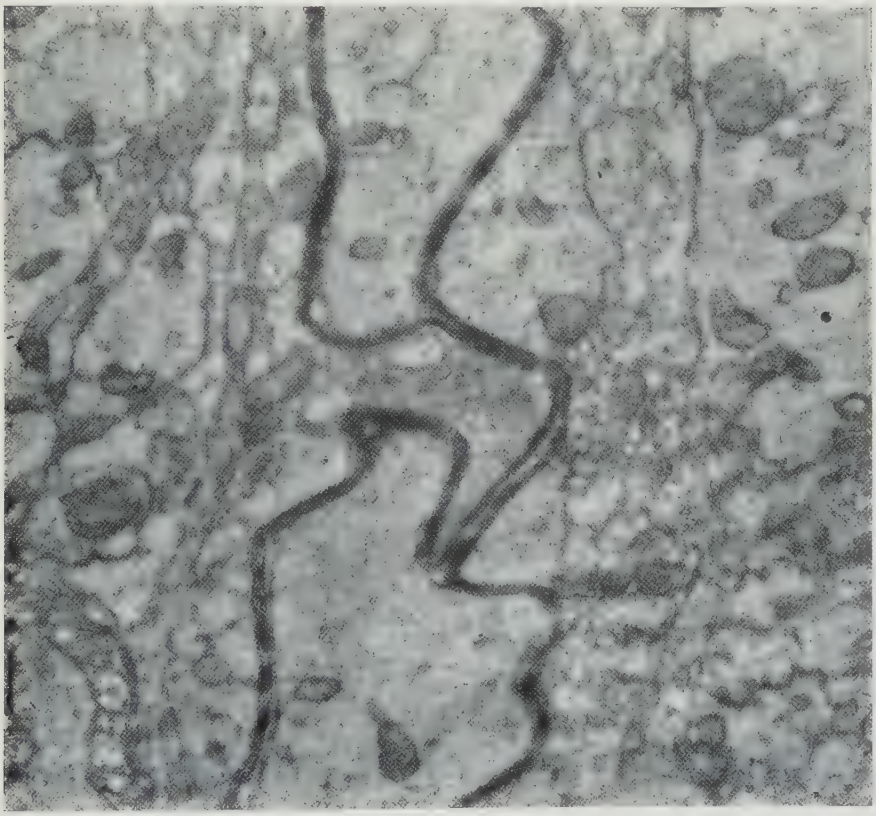

Fig. 1. Fibre myélinisée de la zone sous-épendymaire, en coupe oblique. Grossissement: 22500 ×

niques de la zona externa infundibuli, sur la zone de jonction entre infundibulum et pars tuberalis, enfin sur les capillaires du plexus intercalaire et les anses intra-infundibulaires courtes.

1. Fibres myéliniques. Dans la zone sous-épendymaire s'observent de nombreuses fibres myélinisées, de forme très irrégulière, dont le diamètre varie de 6—7 microns à 0,25 micron environ. L'irrégularité du contour de ces fibres, évidente sur coupes sagittales et obliques (Fig. 1) permet de rendre compte de l'aspect inhabituel de certaines de leurs sections (Fig. 2). Leur gaine, dont l'épaisseur est généralement comprise entre 0,3 micron et 20 millimicrons, peut

comporter jusqu'à une vingtaine de lamelles élémentaires, mais le nombre de celles-ci peut s'abaisser à 2 ou 3. A l'intérieur de ces fibres on peut observer de petites mitochondries et des chondriocontes à crêtes internes assez nettes.

Fig. 2. Fibres myélinisées de la zone sous-épendymaire. Coupe approximativement transversale. Les fibres amyéliniques peuvent être très fines. Grossissement: 27 000 ×

2. Zona externa infundibuli. La zona externa infundibuli renferme de très nombreuses fibres amyéliniques de calibre irrégulier et très variable. Certaines sont très fines, d'un diamètre de l'ordre de 0,1 et même 0,05 micron (Fig. 2). Des fibres analogues se rencontrent dans la zona fibrillaris, entre les fibres myélinisées et certaines fibres amyéliniques épaisses, renfermant des granules de neurosécrétat (Fig. 3) ou des vésicules vides, de taille voisine de celle des granules de neurosécrétat. Certaines coupes de fibres amyéliniques paraissent contenir des microvésicules de type synaptique; nous nous proposons de réexaminer ce dernier point plus en détail ultérieurement.

3. Zone de jonction entre pars tuberalis et éminence médiane. Dans la zone de jonction entre pars tuberalis et éminence médiane s'observent de nombreux capillaires appartenant au plexus intercalaire ou à des anses intra-infundibulaires courtes (Fig. 4). Ces capillaires sont entourés par une basale relativement épaisse autour de laquelle on rencontre parfois quelques péricytes ou de très fines fibrilles

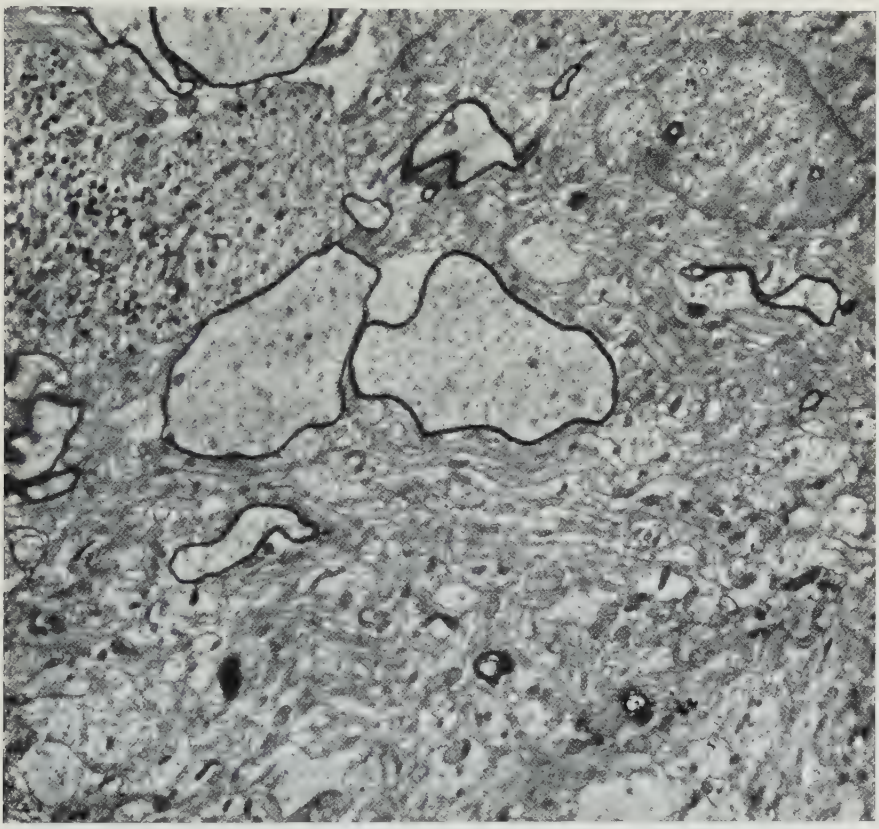

Fig. 3. Zona fibrillaris. Fibres myélinisées de calibre irrégulier, mêlées à des fibres non myélinisées. En haut et à gauche, une fibre amyélinique épaisse renferme des granules de neurosécrétat. Grossissement: 6000 ×

de type collagène. Au contact immédiat des capillaires, tout autour d'eux s'ils sont en plein tissu nerveux, du côté nerveux seulement s'ils sont à la limite de la pars tuberalis, on observe une grande quantité de formations nerveuses amyéliniques, d'un diamètre de l'ordre du micron ou un peu plus (Fig. 5). Ces formations, extrêmement nombreuses, séparées par places par des prolongements plus denses et finement fibrillaires et de nature névroglique probable, renferment d'importants amas de microvésicules de 40 à 60 millimicrons de diamètre, entourées d'une membrane et dont l'aspect et la densité aux électrons sont ceux de microvésicules synaptiques.

4. Capillaires du plexus intercalaire et des anses intra-infundibulaires courtes. Examinés à un grossissement suffisant les capillaires du plexus intercalaire (Fig. 5) et des anses intra-infundibulaires courtes se montrent constitués par un

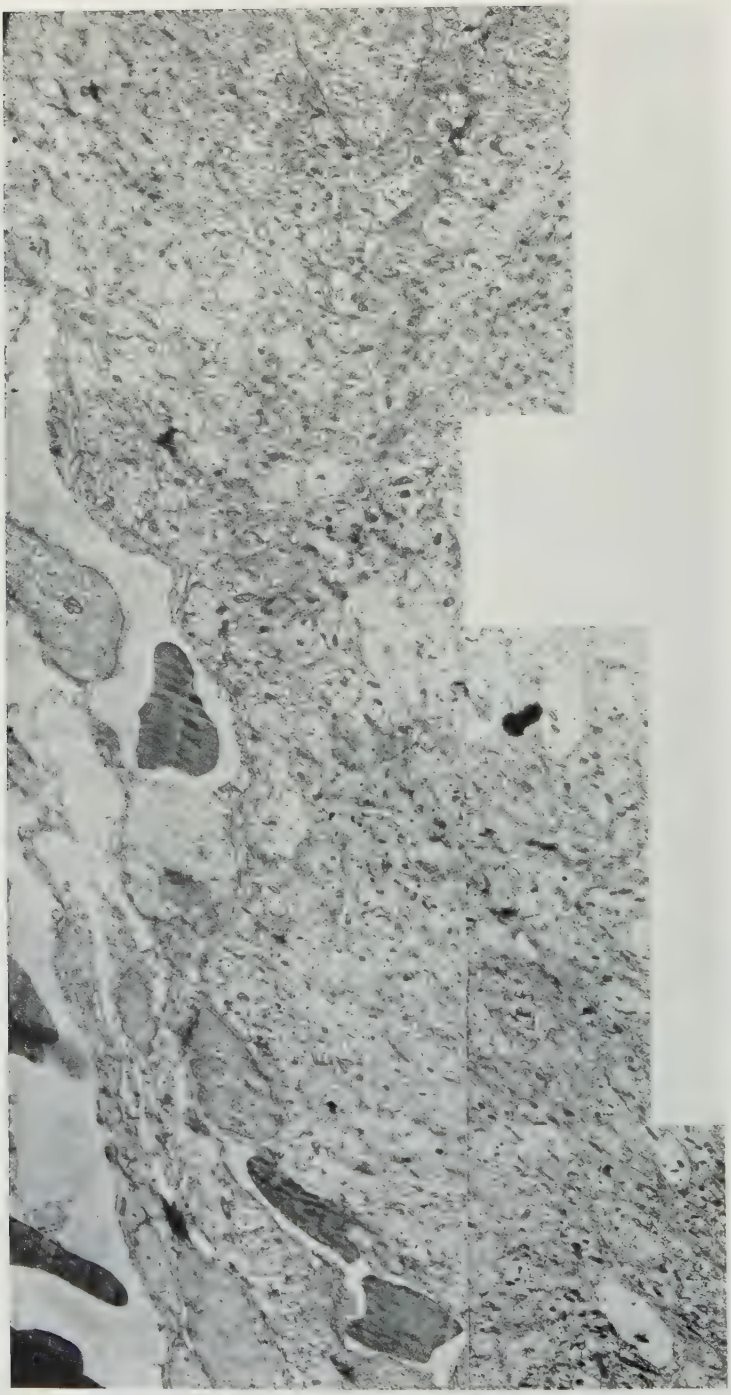

Fig. 4. A gauche, des capillaires appartenant au plexus intercalaire ou à des anses intra-infundibulaires courtes. Ils sont situés entre la pars tuberalis et l'éminence médiane dont on voit une partie à droite. Du coté nerveux de ces capillaires et à leur contact immédiat se rencontrent des formations nerveuses amyéliniques montrant des images qui rappellent les microvésicules synaptiques. Grossissement: 4800 ×

endothélium continu. Les cellules de cet endothélium forment un revêtement extrêmement mince à distance des pericaryons et présentent par places des zones amincies ou des pores d'un diamètre de quelques centaines d'angströms. Ces pores

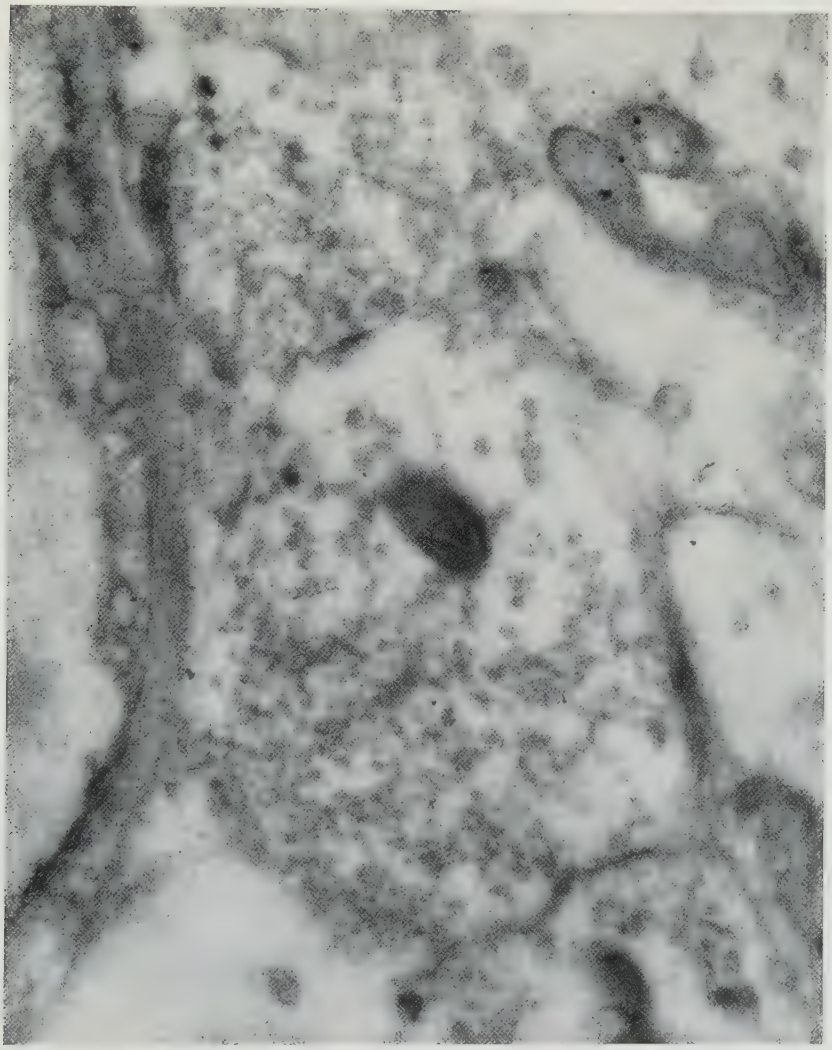

Fig. 5. On remarque à gauche la paroi d'un capillaire, dont l'endothélium, vers le bas, présente des pores, obturés par une membrane très mince. Au contact de la basale du capillaire, des formations amyéliniques renferment des vésicules dont l'aspect, les dimensions et la densité sont ceux des microvésicules synaptiques. Grossissement: 45000 ×

sont diaphragmés par une très mince membrane dont l'épaisseur est de l'ordre d'une centaine d'angströms.

Discussion

Les fibres myéliniques observables au microscope électronique dans la zona fibrillaris correspondent aux éléments déjà signalés chez le cobaye par Spuler (1951), Becker (1955) et Barry et Lenys (1960) à l'aide des techniques classiques

de mise en évidence des fibres myéliniques. Nous ne pouvons décider pour l'instant si certaines de ces fibres sont effectivement de type neurosécrétoire, comme il est admis par divers auteurs, et si elles peuvent être comparées aux fibres neurosécrétoires myélinisées observées au microscope électronique par Duncan (1955) dans la tige infundibulaire chez *Gallus dom.*

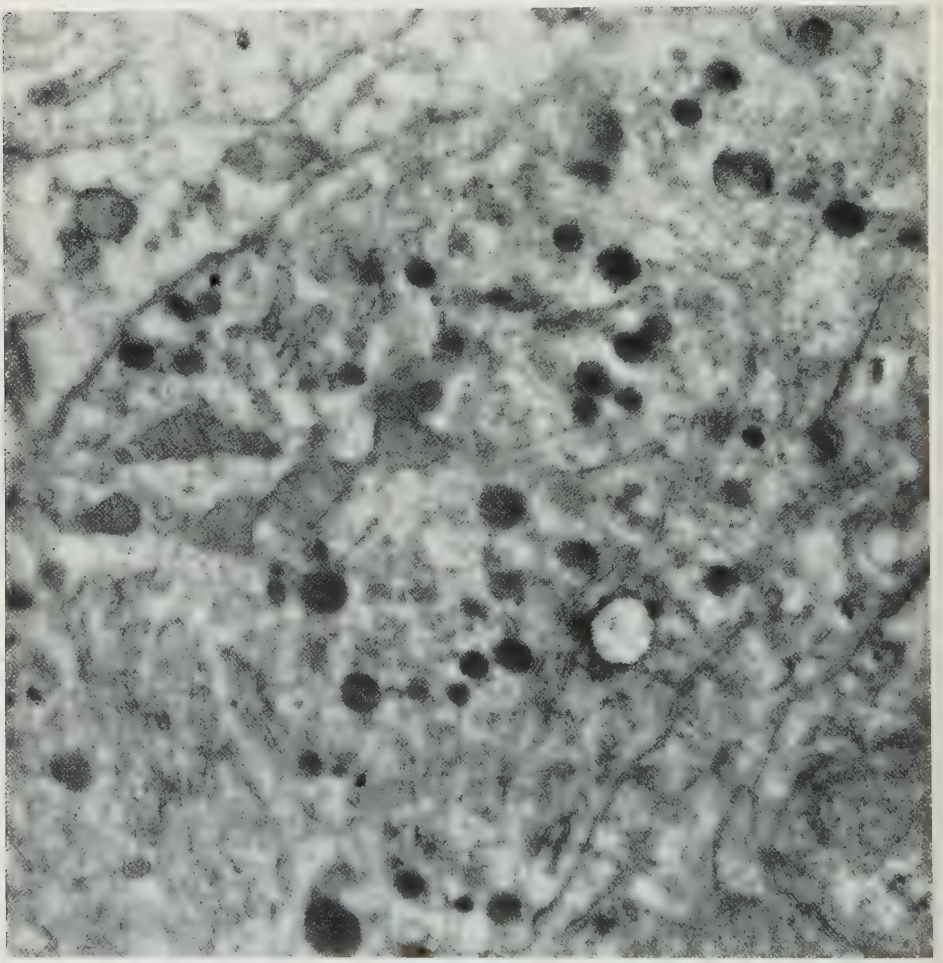

Fig. 6. On voit, en bas et à droite, une partie du noyau d'un péricyte bordant un capillaire du plexus intercalaire ou des anses intra-infundibulaires. Au contact de ces capillaires se rencontrent parfois des fibres amyéliniques neurosécrétoires. Grossissement: 27 000 ×

Le neurosécrétat granulaire présent au niveau de l'éminence médiane chez le cobaye paraît représenté surtout par des vésicules à contenu dense, de diamètre variable, compris entre 150 et 300 millimicrons environ. Dans certains cas il paraît affecter l'aspect de «vésicules pâles» analogues à celles déjà décrites par divers auteurs (Bargmann et Knoop 1957; Fujita 1957; Palay 1957; Hartmann 1958; Green et Maxwell 1959). Cet aspect semble d'ailleurs très difficile à distinguer de celui de «vésicules vides», lié à la cession de neurosécrétat (Hartmann 1958; Gerschenfeld, Tramezzani et de Robertis 1960).

Exceptionnellement nous avons pu observer des fibres amyéliniques neurosécrétoires à proximité ou au contact de capillaires de la zone de jonction (Fig. 6). Cette disposition, qui rappelle les images que nous avons signalées chez le grand Rhinolophe (Barry et Lenys 1960) et celles décrites récemment par Okamoto et Ihara (1960) chez divers Mammifères, correspond à une articulation probablement analogue aux articulations neurosécréto-capillaires observables au niveau des anses intra-infundibulaires longues.

Les rares corps de Herring que nous avons rencontrés au niveau de la zona fibrillaris présentent la structure fine caractéristique bien décrite par de nombreux auteurs: importante accumulation de microgranules, nombreux chondriocontes, matériel neuroplasmique de type granuleux ou cytomembranes etc. . . .

Les fibres amyéliniques fines observables au niveau de la zona externa de l'infundibulum correspondent en majeure partie aux fibres du faisceau tubéro-hypophysaire. Ces fibres, que nous avons proposé de désigner sous le nom de fibres lisses amyéliniques fines (Barry et Lenys 1960) semblent, sur préparations traitées par la technique de Bielschowsky-Gros-Schultze après préfixation dans le mélange AFA, se diriger vers les capillaires du plexus intercalaire et autour des anses intra-infundibulaires (Barry 1960), au même titre que les fibres analogues étudiées chez le chat par Nowakowski (1951) ou que les fibres granuleuses fines étudiées chez la souris par Stutinsky (1955) et Becker (1955). Les formations nerveuses amyéliniques observables en particulier au niveau de la zone de jonction de la pars tuberalis et de l'éminence médiane correspondent, selon toute vraisemblance, à la terminaison de fibres nerveuses. Cette hypothèse nous paraît justifiée non seulement par le diamètre de ces formations et la présence à leur intérieur de microvésicules du type vésicules synaptiques (de Robertis 1953; de Robertis et Bennett 1954; Palade 1954; Palay 1954), mais encore par le fait que la technique de Rasmussen pour la mise en évidence élective des boutons terminaux permet (après modification pour coupes à la congélation) de révéler dans la même région un très grand nombre de terminaisons massuées péricapillaires (Barry 1960, inédit). L'existence, au niveau des capillaires du plexus intercalaire et des anses intra-infundibulaires, d'un espace «sous-endothélial» ou «péri-endothélial» analogue à celui décrit autour des capillaires de diverses glandes endocrines (Monroe 1953; Dempsey et Peterson 1955; Rinehart et Farquhar 1955; Lever 1955a et b; Ekholm et Sjöstrand 1956; Ekholm 1957; Ferreira 1957; Zelander 1957; Belt 1957; Bargmann et Knoop 1960) ou au niveau du lobe nerveux de l'hypophyse (Palay 1955, 1957; Hartmann 1958) conduisent à penser que ces capillaires pourraient être le lieu d'échanges «neuro-humoraux» particuliers. Dans cette perspective les microvésicules synaptiques que nous avons observées au niveau des terminaisons nerveuses péricapillaires, présumées tubéro-hypophysaires, de l'éminence médiane pourraient jouer un rôle assez différent de celui des microvésicules synaptiques observées par divers auteurs au niveau des extrémités neurosécrétoires péricapillaires du lobe nerveux (Hartmann 1958; Gerschenfeld, Tramezzani et de Robertis 1960; de Robertis 1960). Plus précisément nous inclinerions à penser que les données morphologiques précédentes suggèrent un double mécanisme de commande neuro-humorale des activités préhypophysaires, l'une s'exerçant par l'intermédiaire de *substances neurosécrétées* d'origine hypothalamique, l'autre par

l'intermédiaire de *neuro-humeurs* du type des intermédiaires chimiques, le tractus supraoptico-hypophysaire d'une part et le tractus tubéro-hypophysaire d'autre part étant respectivement impliqués dans l'un et l'autre de ces mécanismes.

Résumé

L'examen au microscope électronique de l'éminence médiane du cobaye y révèle la présence:

1. de fibres nerveuses myélinisées très irrégulières dont la nature exacte demeure à préciser,

2. de fibres nerveuses amyéliniques de type neurosécrétoire et de corps de Herring,

3. de fibres nerveuses amyéliniques fines, non sécrétoires,

4. de terminaisons nerveuses amyéliniques périvasculaires, particulièrement abondantes au niveau des capillaires du plexus intercalaire et des anses intra-infundibulaires courtes, ces terminaisons renfermant de nombreuses micro-vésicules de type synaptique,

5. de capillaires à endothélium continu mais présentant des séries de pores diaphragmés par une très mince membrane.

Ces données suggèrent l'hypothèse d'un double mécanisme de commande neuro-humorale des activités préhypophysaires, s'exerçant par l'intermédiaire d'une part de substances neurosécrétées d'origine hypothalamique, d'autre part de neuro-humeurs du type des intermédiaires chimiques.

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Kurze Übersichtstabelle zur Geschichte der Medizin

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INHALTSÜBERSICHT

Primitive Medizin · Heilkunde der alten Kulturvölker: Sumerer, Babylonier, Assyrer. Ägypten. Altjüdische Medizin. Medizin der alten Perser. Altindien. Altchina. Japan. Kulturvölker Altamerikas. Griechen. Römer. Heilkunde im germanisch-keltischen Altertum · Heilkunde des Mittelalters: Heilkunde im byzantinischen Kulturkreis. Arabische Heilkunde. Abendland · Neue Zeit: Erste naturwissenschaftliche Periode der modernen Medizin. Von Vesal bis zum Ausgang der Iatrochemie und Iatrophysik (1500—1700). Zeitalter starken Einflusses der Philosophie auf die Medizin (1700—1830). Zweite naturwissenschaftliche Periode der modernen Medizin (1838—1918). Von der Begründung der Zellenlehre bis zum Sieg des Konstitutionsgedankens · Neueste Zeit (1918—1950) · Personen- und Ortsverzeichnis · Sachverzeichnis.

ZUR INFORMATION

Die 7. Auflage der bekannten Geschichtstabelle erscheint in völlig neuem Gewande und im Umfang wesentlich dadurch erweitert, daß die wichtigsten Daten der historischen Entwicklung der Medizin bis zur Mitte des 20. Jahrhunderts fortgeführt wurden. Ferner wurden die biographischen Angaben möglichst durch Zusatz der Geburts- und Sterbedaten vervollständigt und zahlreiche Ergänzungen eingefügt.

Leitmotiv bleibt das Bestreben, dem Studierenden der Medizin, dem Arzt und Forscher eine Übersicht über den Wandel der Grundlagen des ärztlichen Denkens zu geben, damit sie die Medizingeschichte zur Gegenwart in lebendige Beziehung setzen können. Neu einbezogen wurden die Zahnheilkunde sowie die ärztlichen Standesprobleme in Deutschland. Darüber hinaus wurde die Mitwirkung der Ärzte an den allgemeinen Zeitfragen berücksichtigt.

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